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## INVITED CRITICAL COMMENT . . .

# A PLEA FOR IMPROVED PRESENTATION OF TYPE MATERIAL FOR COCCIDIA

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**ABSTRACT:** The “true” coccidia (phylum Apicomplexa, suborder Eimeriina) constitute a large and heterogeneous group of parasitic protozoa. Despite the large number of described species (ca. 1,650) and the medical and veterinary importance of some (e.g., *Toxoplasma*), 2 facts are clear: (1) the majority of coccidia species are probably yet undescribed, and (2) the phylogenetic relationships of those described species are poorly known. Contributing to the latter dilemma is the lack of a tradition to provide type specimens by those who describe new species, even though the International Code of Zoological Nomenclature specifically recommends the designation of a type specimen with the description of a new species. With the publication of a new edition of the Code (1985), explicit provisions are made for the unique concerns of taxonomists working with Protozoa. Here we remind those interested in the taxonomy of coccidia of an already established method for preserving oocysts in resin and, as an alternative, suggest the standardization of a photographic procedure through which type specimens of coccidian oocysts might also be submitted to and maintained in accredited museums. Thus, coccidia taxonomists should no longer have an excuse for their failure to designate types.

The protozoan phylum Apicomplexa Levine, 1970, comprises a large and heterogeneous group of obligate intracellular parasites including many species of medical and veterinary significance (e.g., *Plasmodium*, *Babesia*, *Cryptosporidium*, *Eimeria*, and *Toxoplasma*). About one-third of the approximately 4,600 described species in the phylum are placed in a single family, Eimeriidae, and the vast majority of these species are known from a single life-cycle stage, the sporulated oocyst. It is the process by which new eimeriids are described that we wish to address in this essay.

### HISTORICAL PERSPECTIVE

Although the *names* of apicomplexans reflect the use of the International Code of Zoological Nomenclature, the taxonomic *procedure* followed in documenting the existence of new eimeriid species has not been consistent with the intention of the Code. For example, the Code explicitly recommends the designation of type specimens for new species, but a type tradition is lacking among taxonomists working with the Eimeriidae.

Historically, under the Zoological Code, “the type is a specimen,” with the implication that the type specimen will be available for future study. Unfortunately, most scientists describing new coccidian species have not devoted attention

to developing methods to preserve coccidian oocysts permanently or to other viable alternatives. Consequently, reference collections of preserved specimens of eimeriid species do not exist, with but few exceptions (e.g., *Calyptospora empristica*, see Fournie et al., 1985).

According to the most recent edition of the Zoological Code (Ride et al., 1985), a type specimen serves as “the objective standard of reference by which the application of the name it bears is determined, no matter how the boundaries of the taxon may change” (Article 61 (a), p. 115). Thus, the type specimen is intended to be unchanging and objective, whereas the limits of a nominal species are recognized to be subjective and transient. The type specimen, therefore, serves as an anchor for the name, and to some extent, it *is* the name (see Mayr et al., 1953).

The Bacteriological Code of Nomenclature (Lapage et al., 1975) allows, and indeed encourages, the submission of cultures to a type culture collection. Apicomplexans cannot be cultured axenically, and although some eimeriid species have been cultured in host cells, the technical difficulties in culturing new species isolated from wild animals preclude the routine submission of type cultures.

Both the Botanical Code (Voss et al., 1983; Article 9.3) and the Bacteriological Code (Rule 18A) have made provisions for organisms that cannot be permanently preserved: drawings are acceptable as type specimens. Previous editions of the Zoological Code have allowed illustrations

to serve as lectotypes, but have not addressed the issue of whether illustrations may serve as holotypes. In the most recent version of the Zoological Code (1985, Article 73 (a) (iv), p. 149) the following statement is made, "Designation of an illustration of a single specimen as a holotype is to be treated as designation of the specimen illustrated; *the fact that the specimen cannot be traced does not of itself invalidate the designation* (emphasis ours)." We interpret this to mean that illustrations are finally acceptable substitutes for type specimens. According to Article 72 (c) (v), p. 145, "... in the case of a nominal species group taxon based on an illustration or description . . . , the specimen illustrated or described and not the illustration or description" (is eligible as a name-bearing type). Regardless of whether the specimen or the illustration is considered the type, it seems clear that the *intent* of the new edition of the Zoological Code is to provide mechanisms by which type specimens can be identified for more nontraditional animal groups (e.g., protozoa). Whether we call them holotypes, syntypes, or even phototypes (=iconotype, see Frizzell, 1933) is of little concern to us. The major point of this essay is that we use the technology available to begin a type specimen tradition for the coccidia.

Given our conclusion that illustrations may be considered legitimate replacements for type specimens under the Code, an additional problem arises for biologists working on the coccidia. For an illustration to serve as a type, there is an implicit requirement that the illustration be based on a single individual. Types serve as the "last court of appeal" in disputes over the application of scientific names (Mayr et al., 1953). It is imperative that an illustration intended to serve as a type specimen represent a single individual because of the danger of basing a composite illustration on 2 or more species, a situation that would become a nomenclatural nightmare. Yet, composite drawings of sporulated oocysts are the standard form of presentation of taxonomic findings in descriptions of new eimeriid species. This is not meant to imply that composite illustrations have no value in coccidian taxonomy; rather, this very useful means of presenting taxonomic information should be supplemented by material less subject to errors of interpretation.

The Zoological Code does not explicitly address the question of the validity of names established without the designation of type specimens. Some authors (see Blackwelder, 1967, pp.

165-166) would consider such names invalid. Under such a strict interpretation of the Code, the status of most of the species names in the Eimeriidae would be uncertain. Indeed, the same taxonomic practices are undoubtedly widespread among taxonomists working on other apicomplexans; we are restricting our comments to a single family with which we are most familiar. Our objective in writing this essay, however, is not to threaten to declare hundreds of species names invalid, but to draw attention to the lack of a standard for the description of new eimeriids and to emphasize how this has impeded efforts to understand the systematic relationships among the genera and species within the Eimeriidae. Systematics, or "beta taxonomy" can only exist with a firm foundation of "alpha taxonomy" (and nomenclature) to support it. If there is no means of comparing species (i.e., no reference collections), neither phylogenetic nor phenetic relationships among species can be discerned. The literature on these organisms then can be only an endless series of descriptions and redescrptions, with valueless speculation regarding the significance of real or imagined differences.

The uses of type specimens go beyond their importance in nomenclature, however, and thus the lack of a type tradition among biologists working with the coccidia has implications beyond the invalidity of the species names. Blackwelder (1967, p. 166) identified 3 ways in which type specimens are useful to the scientific community. These uses of types will be considered, using specific examples drawn from the taxonomic literature on apicomplexans.

First, in poorly known groups, type specimens serve as "a source of unchallengeable characters" (Blackwelder, 1967, p. 166) for the group. The monotypic family Spirocystidae Léger and Duboscq, 1915, is sometimes cited as an example of just how little is known about some apicomplexans (Levine, 1982, 1985). The type and only species, *Spirocystis nidula* Léger and Duboscq, 1911, was observed only once by the original authors. Although later attempts to find other examples of this species were unsuccessful, Léger and Duboscq (1915) published their original findings. The meront stage of this organism does not resemble any known coccidian, and the oocysts, with their "vermicular sporozoites," bear a striking resemblance to nematode eggs. In short, there is little in the evidence provided that would allow this organism to be placed in the phylum Apicomplexa with any degree of confidence. If a



type specimen existed, it would be possible to reexamine the organism and check some of the more questionable (or dubious) characters; this, in turn, would allow a reevaluation of the status of *Spirocystis*. In the absence of a type specimen tradition, descriptions such as this one are maintained in the literature because there is no alternative. Unfortunately, the evidence presented for the existence of *Spirocystis* is not much weaker than the evidence presented for the existence of many other coccidian species.

Second, in studies of intraspecific variation (and even interspecific similarities), the type specimen represents "one point which unequivocally falls within the species" (Blackwelder, 1967, p. 166). Presently there are several factors related to intraspecific variation that raise problems in eimeriid identification when studying sporulated oocysts from wild animal populations. Although the variations seen between individuals within a species are not unique to coccidians as organisms (note, for example, the great differences between breeds of dogs), coccidian taxonomists need to be reminded of their existence. For example, oocysts of one eimerian may vary greatly in size, but otherwise be indistinguishable from each other (e.g., oocysts of *Eimeria separata* Becker and Hall, 1931, vary in size by as much as 40% over patency; Duszynski, 1971) or oocysts presumed to represent one species may be highly polymorphic within the same or closely related host species (e.g., *Eimeria reichenowi* Yakimoff and Matschoulsky, 1935; Parker and Duszynski, 1986). In addition, the role of evolution in the morphologic similarity of different species in the same host (e.g., chicken eimerians; Joyner, 1982) and the fact that some species can infect unrelated host species (e.g., *Eimeria chinchillae* De Vos and Van der Westhuizen, 1968, see De Vos, 1970; *Eimeria tamiasciuri* Levine and Ivens, 1965, see Vance and Duszynski, 1985, and Hill and Duszynski, 1986) must be recognized as confounding factors when describing new coccidian species from sporulated oocysts from wild animals. The existence of a type specimen tradition, especially if large reference collections of voucher specimens were available for study, would greatly aid our dealing with such complicating factors.

Finally, the third way in which type specimens can be useful to the scientific community is that types serve as a means of checking the accuracy of published descriptions (Blackwelder, 1967, p. 166). This is in some ways comparable to the

replication of an experiment in another laboratory, and it serves as a supplement to the peer review process.

The importance of type specimens (and indeed of taxonomy) goes beyond their necessity in systematics. Good taxonomy is an integral aspect of the scientific method for experimental biologists. In order to draw general conclusions from an experiment it is necessary to know that the organisms under study represent a homogeneous group. All other investigations of eimeriid coccidia, whether biochemical, physiological, immunological, etc., are undermined by a shaky taxonomic foundation.

### PROPOSED SOLUTIONS

What then can be done to promote progress in eimeriid taxonomy and systematics? The publication of the newest edition of the International Code of Zoological Nomenclature (Ride et al., 1985) should usher in a new era in protozoan systematics. Historically, the Zoological Code has been inadequate for these organisms and, as a consequence, has been applied in a rather haphazard fashion. Now, for the first time, explicit provisions are made for the unique concerns of taxonomists working with protozoa. For the new provisions in the Zoological Code to be put into practice, we must require a reevaluation of the taxonomic procedures used with groups such as the Eimeriidae. Perhaps of greatest importance, then, is the need to create an awareness among biologists working with eimeriids of the value of designating type specimens. Building a type tradition will require not only the designation of holotypes for new species, but also the designation of lectotypes or neotypes for existing names. In addition, type *species* need to be designated for the known genera.

Methods of permanent preservation of coccidian oocysts now exist (Marchiondo and Duszynski, 1978, 1988), so there is no longer any reason not to begin to designate type specimens for those species that can be handled in this manner. In addition, the new provisions of the Zoological Code regarding illustrations provide an alternative: photography offers many of the advantages of preserved specimens at a fraction of the cost of the methods employed by Marchiondo and Duszynski (1978). Thus, when describing new species one could prepare a series of photomicrographs, chosen carefully to illustrate as many features of the new species as possible, and perhaps mounted on poster board with the nec-

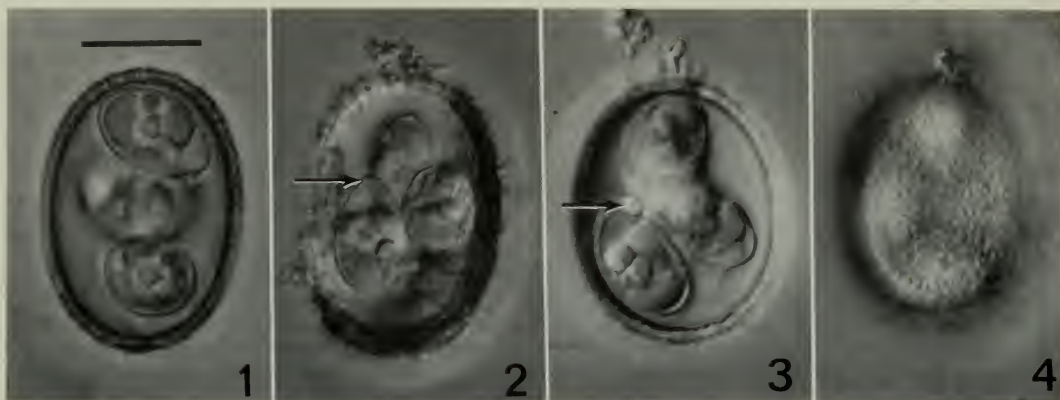


FIGURE 1. Suggested format for beginning a type specimen tradition among coccidiologists. A plate of photomicrographs of representative oocysts showing main distinguishing features of each new species.

essary structural, host, and locality data on the back (Figs. 1, 2). If we standardize the size, the poster can be submitted to, and maintained in, accredited museums just as slides of helminth types are catalogued currently. Two copies of the phototypes should probably be submitted so that one set remains permanently in the repository while the other would be on loan. Ideally, hapantotypes, composite types in which all stages in the life history are represented, should be submitted as advocated by Williams (1986). For many coccidia spp. this would be next to impossible, however, and the deposition of a syn-

type (=phototype) series consisting of oocysts probably represents a more realistic goal. If reputable journals made the designation of type specimens (including type species for new genera) a requirement for publication (as most already do for helminths), there would no longer be an excuse for the failure to designate types. Type specimens are not a panacea: there is some information that cannot be obtained from preserved specimens or photomicrographs. Indeed, there is much to be gained by the collection of fresh material. However, type specimens represent a point of common ground for discussion,

**DATE SUBMITTED:** 5 August 1987

**COCCIDIAN SP:** *Eimeria tadarida*

**TYPE HOST:** *Tadarida femorosacca* (Merriam)

**DEPOSITED IN MUSEUM?:** Museum of Southwestern Biology  
Division of Mammals  
The University of New Mexico  
Albuquerque, NM 87131

**MUSEUM #:** MSB #53835 (female)

**COLLECTOR, #, DATE:** J. Haydock, #282, 27 October 1980

**TYPE LOCALITY:** MEXICO, Sonora: 19.3 km E Alamos by road Rio Cucujaqui

**OTHER COCCIDIANS PRESENT?** None

**PREVALENCE:** 1 of 1 from Sonora; 0 of 17 from Baja California Norte, MX

**OTHER HOSTS WITH THIS SP:** None to date

**NATIONAL MUSEUM #:**

**REFERENCE OF ORIGINAL DESCRIPTION:** Duszynski, D. W.,  
D. W. Reduker and B. B. Parker, 1988. *Eimeria* from bats of the world. II. A new  
species in *Tadarida femorosacca* from Sonora, Mexico. *Journal of Parasitology* 74:000-000.

**LEGEND FOR FIGURES OF SYNTYPES.** X 1600. Bar = 10  $\mu$ m.

1. Note striated appearance of oocyst wall. 2. Note tiny Stieda body (arrow) and vacant  
space below pointed end of sporocyst (also seen in 1 and 3) that is probably a substieda  
body. 3. Note fragments (arrow) that may be 1 of 3 polar bodies or part of fragmented  
oocyst residuum. 4. Mammillated outer wall of oocyst.

FIGURE 2. Suggested format for beginning a type specimen tradition among coccidiologists. Proposed data sheet that could be attached to the back of the posterboard with the representative oocysts. This poster, in some standardized size (e.g., 15  $\times$  22.5 cm), could be submitted to accredited national museums for all new species descriptions.



and only when the designation of type specimens is mandatory can the current state of eimeriid systematics change. Biologists working with the Eimeriidae would do well to heed the advice of Ferris (1928, p. 105): "The proper aim [of taxonomy] is not to name species, but to know them."

### ACKNOWLEDGMENTS

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## EFFECT OF PAIRING *IN VITRO* ON THE GLUTATHIONE LEVEL OF MALE *SCHISTOSOMA MANSONI*

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**ABSTRACT:** The effect of *in vitro* incubation on the level of the intracellular nucleophile, glutathione (GSH), in adult *Schistosoma mansoni* was investigated. The GSH levels of freshly collected adult male and female parasites were  $8.5 \pm 2.5$  and  $2.7 \pm 0.7$  nmol/10 worms, respectively, as determined by an enzymatic assay. Twenty-four-hour incubation of unpaired males in RPMI-1640 medium at 37 C resulted in a 1.7-fold increase ( $P < 0.001$ ) in GSH level that remained elevated for at least 7 days. The increase was dependent on exogenous L-cystine, suggesting that it was due to biosynthesis of GSH. Biosynthesis in male *S. mansoni* was confirmed by isolating [ $^3\text{H}$ ] GSH from parasites incubated in medium containing L-[ $^3\text{H}$ ] cystine or [ $^3\text{H}$ ] glycine. In contrast to unpaired males, the GSH level of paired males as well as that of unpaired or paired females did not increase after 24 hr *in vitro*. When males that had been incubated unpaired for 24 hr were allowed to couple *in vitro* with freshly collected females, their GSH level fell to that of continuously paired males. These observations provide evidence that *in vitro* female schistosomes can influence the physiology of the male.

Glutathione (L- $\gamma$ -glutamyl-L-cysteinylglycine, GSH) is the major intracellular thiol in animals, plants, and microorganisms. This tripeptide, which is usually present at millimolar concentrations, is synthesized from its constituent amino acids in a 2-step, ATP-dependent pathway. GSH participates in a plethora of biochemical processes, acting as a cofactor in the synthesis of proteins and deoxyribonucleotides, as a reducing agent in disulfide-sulfhydryl exchange reactions, a  $\gamma$ -glutamyl donor in amino acid transport, and in protection against cellular damage due to radiation, reactive oxygen species, and toxic chemicals of both endogenous and xenobiotic origin (for reviews see Larsson et al., 1983; Meister and Anderson, 1983).

Of these various functions, the role of GSH as a nucleophile in the detoxication of xenobiotics is one of the best characterized. Reaction of the thiolate anion ( $\text{GS}^-$ ) with an electrophile to form a water-soluble thioether conjugate is catalyzed by a family of enzymes, the GSH S-transferases (E.C. 2.5.1.18) (Mannervik, 1985). These enzymes are widely distributed in nature, being found in both vertebrates and invertebrates, including metazoan parasites (Morello et al., 1982; Kawalek et al., 1984; Jaffe and Lambert, 1986; O'Leary and Tracy, 1988).

Our laboratory is studying conjugation of xe-

nobiotics with GSH as the prototype drug detoxication pathway in schistosomes. We have found that adult *Schistosoma mansoni* contain at least 4 forms of cytosolic GSH S-transferase (O'Leary and Tracy, 1988) that catalyze the conjugation of a number of model xenobiotics and dichlorvos, the active form of metrifonate. As a prerequisite to studies designed to explore the metabolic fate of GSH conjugates within schistosomes, we first sought to estimate the steady-state GSH levels in adult *S. mansoni* and to assess the effect of *in vitro* incubation on those levels. We report here that after 24 hr *in vitro*, the GSH content of unpaired males increases significantly, but GSH levels of paired males as well as paired or unpaired females do not. Furthermore, the increase in GSH level of unpaired males can be reversed by pairing *in vitro*.

### MATERIALS AND METHODS

#### Chemicals

D-glucose, L-methionine, L-cystine, type III glutathione disulfide (GSSG), 5,5'-dithiobis-(2-nitrobenzoic acid), NADPH,  $\gamma$ -L-glutamyl-L-glutamate, iodoacetic acid, and glutathione reductase (E.C. 1.6.4.2, type III from bakers' yeast), were obtained from Sigma Chemical Co. (St. Louis, Missouri). RPMI-1640 and antibiotic/antimycotic solution (100 U penicillin, 100  $\mu\text{g}$  streptomycin, and 0.025  $\mu\text{g}$  amphotericin B/ml) were purchased from GIBCO Laboratories (Grand Island, New York). A custom preparation of RPMI-1640 without L-cystine, L-methionine, or GSH also was obtained from GIBCO. N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES), 3-(N-morpholino)propane sulfonic acid (MOPS), and 1-fluoro-2,4-dinitrobenzene were products of Research Organic Inc. (Cleveland, Ohio). Bovine serum albumin was pur-

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chased from Miles Laboratories (Elkhart, Indiana). 2-Vinylpyridine and triethanolamine were products of Aldrich Chemical Co. (Milwaukee, Wisconsin), and 5-sulfosalicylic acid was bought from Fisher Chemical Co. (Fair Lawn, New Jersey). L-[3,3'-<sup>3</sup>H] cystine (1.5 Ci/mmol) was a product of Amersham Corp. (Arlington Heights, Illinois). [2-<sup>3</sup>H] glycine (53.0 Ci/mmol) was obtained from DuPont NEN Products (Boston, Massachusetts).

### Parasites and host

Female outbred mice (18–22 g, CF<sub>1</sub> strain, Sasco, Madison, Wisconsin) were exposed subcutaneously (Peters and Warren, 1969) to 200 freshly shed cercariae of a Puerto Rican strain of *Schistosoma mansoni*. Seven to 8 wk later, parasites were recovered by portal venous perfusion (Duvall and DeWitt, 1967) with ice-cold RPMI-1640 containing 25 mM HEPES buffer, pH 7.4. To allow collection of worm pairs, mice were perfused with medium at 37 C. After quickly rinsing 2–3 times with perfusion-temperature medium the parasites were washed briefly with 25 ml RPMI-1640 containing 1% (v/v) antibiotic/antimycotic. Separated worms were washed twice with medium at 25 C and then twice with medium at 37 C. Pairs were washed with medium at 37 C only. The temperature of the wash medium had no measurable effect on parasite GSH content.

### In vitro incubations

Parasites were incubated in 6-well tissue culture plates (Becton/Dickinson, Lincoln Park, New Jersey) at 37 C under an atmosphere of 5% CO<sub>2</sub> in air. To each well, containing 5.0 ml RPMI-1640 supplemented with 25 mM HEPES, 10.0 mg/ml D-glucose, 2.0 mg/ml bovine serum albumin, and 1% (v/v) antibiotic/antimycotic, pH 7.4 (Newport and Weller, 1982), were added 10 unpaired males, 30 unpaired females, or 20 worm pairs. After incubation, groups of parasites were collected and prepared for GSH analysis as described below. The presence of antibiotic/antimycotic had no effect on the values obtained. Sulfur amino acid-deficient RPMI-1640 was similarly prepared. For replenishment experiments, the deficient medium was supplemented with 200  $\mu$ M L-cystine and/or 100  $\mu$ M L-methionine, concentrations of the complete medium. For incubations longer than 24 hr, 70–80% of the culture medium was replaced daily with fresh medium.

For *in vitro* pairing experiments, 20 unpaired males were incubated, as above, for 24 hr. Then, 20 freshly collected females were added to the culture wells. Thereafter, those males that formed pairs were collected and separated from the females prior to GSH analysis. For comparison, groups of unpaired males and continuously paired males were incubated in parallel.

### Sample preparation

Groups of parasites (10 males or 30 females) were removed from the incubation medium, gently blotted on Whatman No. 50 filter paper, and immediately placed in 1.5-ml microcentrifuge tubes (Kontes, Evanston, Illinois) containing 100  $\mu$ l of 5% 5-sulfosalicylic acid. Pairs were first placed in culture medium at 4 C for about 10 min to facilitate separation of the sexes,

and if required, were gently teased apart with camel hair brushes. The parasites were homogenized with a battery powered, hand-held homogenizer (Kontes), and centrifuged at 4 C for 5 min at 20,000 g in a Sorvall RC-5C centrifuge (DuPont Co., Wilmington, Delaware). The supernatant was removed and stored at –20 C for no more than 7 days before GSH analysis. This period of storage had no effect on the values obtained.

### GSH analysis

GSH levels were estimated by a modification of an enzymatic recycling assay (Tietze, 1969). To each of 2 cuvettes were added 100 mmol MOPS buffer, pH 7.0, 40 nmol 5,5'-dithiobis-(2-nitrobenzoic acid), 100 nmol NADPH, and 10  $\mu$ l acid-soluble parasite fraction or 0.125–1.000 nmol GSSG standard plus 10  $\mu$ l of 5% 5-sulfosalicylic acid, in a total volume of 980  $\mu$ l. Cuvettes were warmed to 30 C for 4 min and the reaction was started by the addition of 20  $\mu$ l (0.12 units) glutathione reductase (prepared according to Eyer and Podhradsky, 1986). Enzyme was omitted from the reference cuvette. The rate of color formation at 412 nm was measured with a Gilford Response spectrophotometer (Ciba-Corning, Oberlin, Ohio), using the value  $\epsilon = 13,600 \text{ M}^{-1} \text{ cm}^{-1}$  (Ellman, 1959). Each sample was assayed in triplicate and the GSH level determined by comparison to a standard curve, which was included with each group of samples. GSH level is expressed as the mean  $\pm$  SE of the total of GSH and GSSG, in terms of reduced equivalents per 10 worms. All data were statistically analyzed by Student's *t*-test.

To determine the fraction of total GSH present as GSSG, 50–80 males were homogenized in 200  $\mu$ l of 5% 5-sulfosalicylic acid and the 20,000 g supernatant collected as before. To measure GSSG only, a 100- $\mu$ l aliquot of the supernatant was treated with 2.0  $\mu$ l of 2-vinylpyridine, followed by 6.0  $\mu$ l of triethanolamine (Anderson, 1985) before being assayed. Total GSH was quantitated, after diluting a 20- $\mu$ l sample with 60  $\mu$ l of 5% 5-sulfosalicylic acid.

### Isolation of [<sup>3</sup>H] GSH

In order to demonstrate GSH biosynthesis in male *S. mansoni*, parasites were incubated in sulfur amino acid-deficient RPMI-1640 with additions of 100  $\mu$ M L-cystine and 100  $\mu$ M L-methionine. To separate portions of this medium were added L-[<sup>3</sup>H] cystine (10  $\mu$ Ci/ml) or [<sup>3</sup>H] glycine (10  $\mu$ Ci/ml), and to wells containing 5.0 ml of such media were added 25 freshly collected males. After a 3-hr incubation, worms were collected as before, except that 25 males were homogenized in 250  $\mu$ l of ice-cold 10% perchloric acid. A 200- $\mu$ l sample of the supernatant was derivatized with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene (Fariss and Reed, 1983), with  $\gamma$ -L-glutamyl-L-glutamate included as an internal standard. Gradient separation at 1.0 ml/min was performed on a 5- $\mu$ m Spherisorb NH<sub>2</sub> column (4.6 mm I.D.  $\times$  25 cm; Rainin Instruments Co., Woburn, Massachusetts) using a Gilson high-pressure liquid chromatograph (Gilson Medical Electronics, Middleton, Wisconsin) essentially as described by Fariss and Reed (1983). Column effluent was monitored at 365 nm with a Kratos Model 757 dual-beam UV detector (Kratos, Ramsey, New Jersey). Fractions (0.4 min) were collected from the detector



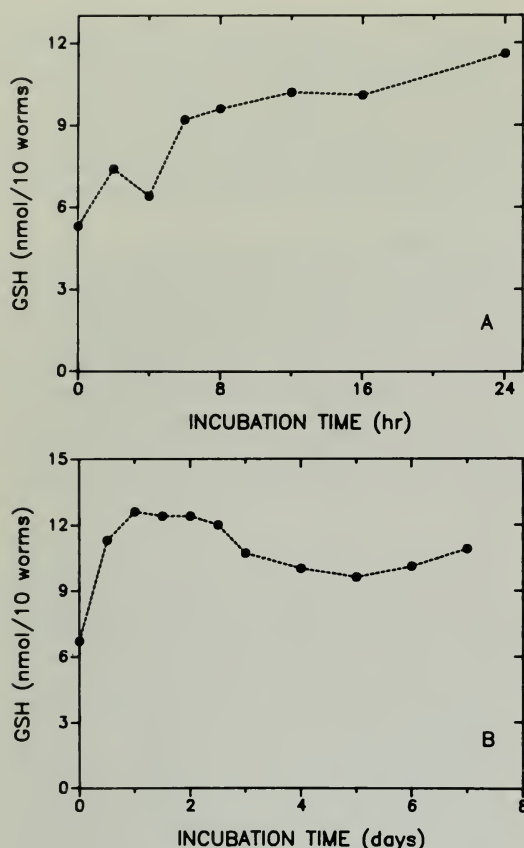


FIGURE 1. Typical time dependence of the *in vitro* GSH level of unpaired male *S. mansoni*. Parasites were collected by perfusion with RPMI-1640 at 4 C. Groups of 10 unpaired males were incubated in 5.0 ml modified RPMI-1640 at 37 C in 5% CO<sub>2</sub> in air. At times indicated, groups were taken for GSH analysis as described in Materials and Methods. The GSH content increased over 24 hr (panel A) and remained elevated for at least 7 days (panel B). For incubations over 24 hr, the medium was changed daily. Each point represents the average of triplicate determinations performed on 1 group of parasites. Variation in the GSH assay was less than 10%.

outlet and radioactivity in each was determined by counting twice in 5.0 ml of Optifluor scintillation cocktail (Packard, Downers Grove, Illinois), using a 2000CA Tri-Carb Liquid Scintillation Analyzer (Packard) and tSIE external standard quench monitoring. All samples displayed the same quench. Unquenched <sup>3</sup>H efficiency was 65%.

## RESULTS

### GSH level in freshly collected worms

Adult *S. mansoni* collected by perfusion with medium at 4 C were kept on ice during the 0.5–1.5 hr required for sample preparation. The GSH

TABLE I. GSH levels of unpaired male *S. mansoni* incubated in RPMI-1640 with or without sulfur amino acids.\*

Parasite preparation	GSH level (nmol/10 worms)
Initial	7.0 ± 0.4
24-hr incubations	
Complete medium	10.9 ± 0.2
S-deficient medium	
– Cys., – Met	7.3 ± 0.1
+ Cys., – Met	12.4 ± 0.2
– Cys., + Met	7.1 ± 1.2
+ Cys., + Met	12.2 ± 0.1

\* Unpaired male *S. mansoni* were collected from murine hosts by perfusion with RPMI-1640 at 4 C. Some were immediately prepared for GSH analysis (initial), whereas other groups were incubated for 24 hr in complete RPMI-1640 or in sulfur amino acid-deficient RPMI-1640 with (+) or without (–) additions of L-cystine (Cys.) and L-methionine (Met) at concentrations of the complete medium (200 μM and 100 μM, respectively). All GSH levels represent the mean ± SE of 3 separate samples.

level of such freshly collected male parasites was found to be  $8.5 \pm 2.5$  nmol/10 worms ( $n = 35$ ), whereas that of females was  $2.7 \pm 0.7$  nmol/10 worms ( $n = 15$ ). GSSG, measured in males only, comprised  $1.4 \pm 0.4\%$  of total GSH ( $n = 4$ ).

### Effect of incubation on the GSH level of unpaired males

Figure 1A illustrates a typical 24-hr time course, during which GSH level approximately doubled in unpaired male schistosomes. Long-term incubation (Fig. 1B) indicated that GSH level was maximal by 24 hr and remained elevated for at least 7 days. Results compiled from 28 separate experiments showed that the mean GSH level of unpaired males increased from  $7.3 \pm 0.9$  nmol/10 worms at time zero to  $13.3 \pm 1.0$  nmol/10 worms after 24 hr incubation. This 1.7-fold increase was statistically significant ( $P < 0.001$ ). GSSG comprised  $1.1 \pm 0.5\%$  of the total at 24 hr ( $n = 4$ ) and was not different from the percentage determined in freshly recovered parasites ( $P > 0.5$ ). In contrast to the increased GSH level observed in complete medium, the GSH level of unpaired males was unchanged after 24 hr in RPMI-1640 depleted of sulfur amino acids (Table I). Other groups of unpaired males were incubated in sulfur amino acid-deficient medium, with additions of 200 μM L-cystine and/or 100 μM L-methionine. The data in Table I demonstrate that the addition of L-cystine, but not L-methionine, at concentrations of the complete medium restored the increase.



### Biosynthesis of GSH

The fact that exogenous L-cystine was required for the observed GSH increase suggested that this increase might be due to GSH biosynthesis. To demonstrate GSH biosynthesis in male *S. mansoni*, groups of unpaired males were incubated in medium containing L-[ $^3\text{H}$ ] cystine or [ $^3\text{H}$ ] glycine. Figure 2A shows a typical HPLC analysis of male schistosomes after 3 hr *in vitro*. The retention times of GSH, the internal standard  $\gamma$ -L-glutamyl-L-glutamate, and GSSG are indicated. Figure 2B and 2C represent the radioactivity detected in fractions, collected from the detector outlet, of samples prepared from parasites incubated for 3 hr in L-[ $^3\text{H}$ ] cystine and [ $^3\text{H}$ ] glycine, respectively. In each case, the retention time of the single major radioactive component was identical to that of GSH. In experiments not illustrated here, the identity of the peak was confirmed to be GSH, by showing that it was composed of equimolar amounts of glutamate, cysteine, and glycine.

### GSH level of paired and unpaired male and female schistosomes

Schistosome pairs were incubated for 24 hr, and the GSH level of males and females determined after physical separation (Fig. 3). GSH levels were also measured in groups of unpaired males and unpaired females incubated in parallel. Unlike unpaired males, the GSH level of paired males remained unchanged over 24 hr ( $P > 0.2$ ). Likewise, the GSH level of females after 24 hr *in vitro*, whether paired or unpaired, was not significantly different from initial levels ( $P > 0.2$ ).

### Effect of pairing *in vitro* on the GSH level of male schistosomes

Freshly collected females were added to equal numbers of unpaired males that had been incubated for 24 hr. In most cases, about 80% of the parasites formed pairs within the next 24 hr. Over the next 3 days, the GSH level was measured in groups of males that had remated. GSH level was also determined in control groups of continuously paired and unpaired males incubated in parallel. As previously observed, the GSH level of unpaired males not only increased and remained elevated throughout the experiment, but was clearly greater than that of continuously paired males (Fig. 4). Strikingly, within 24 hr, the GSH level of remated males decreased to that of continuously paired males. In fact,

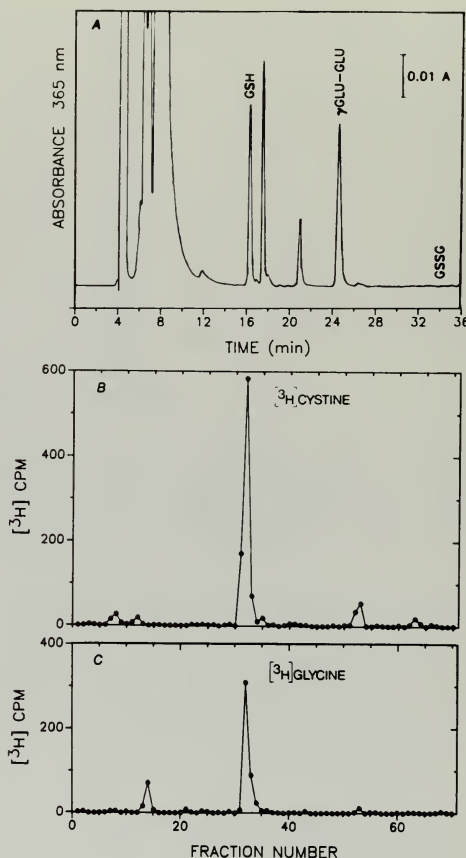


FIGURE 2. Synthesis of [ $^3\text{H}$ ] GSH by male *S. mansoni* after incubation in medium containing L-[ $^3\text{H}$ ] cystine or [ $^3\text{H}$ ] glycine. Unpaired males were incubated in sulfur amino acid-deficient medium supplemented with 100  $\mu\text{M}$  L-methionine and 100  $\mu\text{M}$  L-cystine, to which was added L-[ $^3\text{H}$ ] cystine or [ $^3\text{H}$ ] glycine (10  $\mu\text{Ci/ml}$ ). GSH was analyzed by HPLC as described in Materials and Methods. Panel A shows a typical chromatogram, monitored at 365 nm. Indicated are the peaks for GSH, the internal standard  $\gamma$ -L-glutamyl-L-glutamate, and GSSG. Below is the radioactivity in fractions of samples prepared from parasites incubated for 3 hr in the presence of L-[ $^3\text{H}$ ] cystine (panel B) or [ $^3\text{H}$ ] glycine (panel C), illustrating that the parasites can synthesize GSH. The small radioactive peaks (fractions 13, 17, 18, and 53) represent DNP-amino acid derivatives. Quench was constant in all fractions.

throughout the remainder of the experiment, the GSH level of both continuously paired and remated males remained distinctly lower than that of unpaired males.

### DISCUSSION

The amount of GSH measured in freshly collected adult schistosomes undoubtedly includes both parasite GSH and that of host erythrocytes

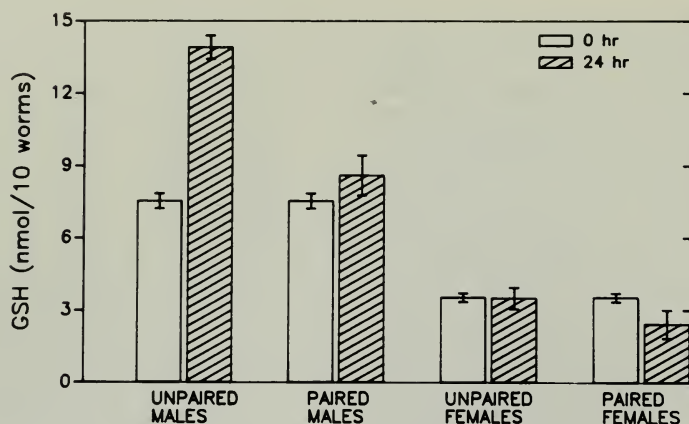


FIGURE 3. Effect of a 24-hr incubation on the GSH level of unpaired and paired male and female *S. mansoni*. Paired worms were collected by perfusion with medium at 37 C and incubated (20 pairs/well) in RPMI-1640. Unpaired parasites were collected and incubated (10 males or 30 females/well) as described in Figure 1. The GSH content of unpaired males increased significantly over 24 hr, whereas that of paired males and paired or unpaired females did not. The data are the mean  $\pm$  SE of 3 separate experiments.

present in the parasite digestive tract. Lawrence (1973) has estimated that an adult male *S. mansoni* contains about  $10^5$  erythrocytes and a female about  $1.4 \times 10^6$  erythrocytes. Given that the

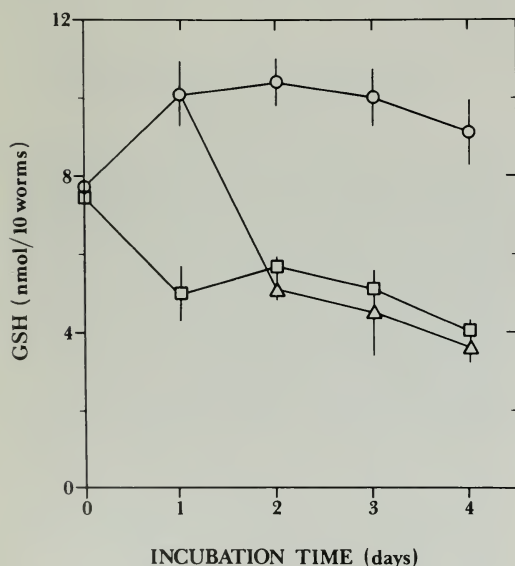


FIGURE 4. Pairing *in vitro* reverses the increase in GSH content of male *S. mansoni*. Unpaired males were cultured for 24 hr in the absence of females, at which time an equal number of freshly collected females were added to the cultures (day 1). Subsequently, those males that had formed pairs (typically 8 of 10) were collected and GSH level determined ( $\Delta$ ). As controls, unpaired ( $\circ$ ) and continuously paired males ( $\square$ ) were incubated and GSH levels determined as described in Materials and Methods. Culture medium in all wells was replaced daily, and each data point represents mean  $\pm$  SE of 3 separate experiments.

mean volume of a mouse erythrocyte is  $4.7 \times 10^{-14}$  L (Jain, 1986) and that an average GSH concentration in mammalian erythrocytes is about 2.7 mM (Tietze, 1969; Fahey et al., 1981; Chappell et al., 1987), one can calculate that host erythrocytes contribute about  $1.3 \times 10^{-11}$  mol or  $<2\%$  of the total GSH in a male worm. Analogous calculations show that the apparent contribution of host erythrocytes to the total GSH in a female worm is  $1.7 \times 10^{-10}$  mol or 65%. However, the latter value is inconsistent with the fact that the GSH content in female schistosomes does not change significantly after 24 hr in the absence of added erythrocytes (Fig. 3), an incubation period 8-fold longer than the time required for complete turnover of the cecal contents (Lawrence, 1973). Thus, the above calculation probably overestimates the actual contribution of host erythrocyte GSH in female schistosomes. Chappell et al. (1987) reported that lysis of erythrocytes in the parasite digestive tract provides GSH for activation of parasite cysteine proteinase. They estimated the concentration of GSH in regurgitate of adult *S. mansoni* to be 4.2 mM. It is difficult to compare their data with ours, because the regurgitate volume was not reported. Furthermore, one cannot exclude the possibility that some fraction of the GSH in the regurgitate may have been released from the parasites by the hypotonic conditions used by Chappell et al. (1987).

To convert the amount of GSH present in schistosomes into concentration, one must know its apparent volume of distribution. Cornford et



al. (1988) have measured the equilibrium water distribution volume of adult *S. mansoni* by a radiometric method. Assuming that GSH is uniformly distributed in total body water, one can estimate that the GSH concentration is about 3.3 mM in freshly collected male worms and 0.8–2.5 mM in females, depending on whether the contribution of host erythrocyte GSH is 65% or 2%, respectively. These concentrations are within the physiological range (0.5–10 mM) found in other organisms (Meister and Anderson, 1983).

The finding that the GSH content of unpaired male *S. mansoni* increases upon *in vitro* incubation was unexpected. However, effects of *in vitro* incubation on GSH levels are not without precedent. For example, Meredith (1986) reported a 30-fold elevation of rat hepatocyte GSH level after 24 hr in modified Williams' Medium E. What is unique about the present study is that alterations of the male schistosome GSH pool were found to be affected by the presence of the female. Effects of the female schistosome on the physiology of the male are apparently unknown, although examples of female dependence on the male are well documented. Maturation of the female reproductive system (Erasmus, 1973), nutrient transfer (Cornford, 1986), and development and feeding of females (Gupta and Basch, 1987) have all been shown to be influenced by the male parasite.

Because increased biosynthesis might be the basis for higher GSH levels, male schistosomes were tested for their ability to synthesize this tripeptide. From experiments summarized in Table I, it is apparent that exogenous L-cystine is essential for the observed increase. This is consistent with the fact that L-cysteine is a precursor of GSH and that the cysteine pool size probably limits the rate of GSH biosynthesis in most cells (Bannai and Tateishi, 1986). The increase was not restored by the addition of L-methionine, suggesting that in male *S. mansoni*, the cystathionine pathway, which converts L-methionine to L-cysteine (Cooper, 1983), is either not present or is insufficiently active to provide adequate amounts of L-cysteine for increased GSH biosynthesis. The existence of the GSH biosynthetic pathway in male schistosomes was confirmed by the incorporation of L-[ $^3\text{H}$ ] cystine and [ $^3\text{H}$ ] glycine from the culture medium into the GSH pool. Recently, Morrison et al. (1987) also showed GSH biosynthesis in male schistosomes using L-[ $^{35}\text{S}$ ] cysteine. When taken together, these results support the idea that the *in vitro* increase of GSH

level in unpaired males results from *de novo* biosynthesis.

We have preliminary evidence, from murine infections in which the number of male worms greatly exceeds the number of females, that the GSH levels of males unpaired *in vivo* is about 2-fold greater than that of paired males (unpubl. obs.). This suggests that even in the mammalian host, male GSH level is affected by the presence or absence of a female in the gynecophoral canal. If confirmed, this observation would provide an *in vivo* correlate to the *in vitro* phenomenon described here.

There are several ways in which this phenomenon might be explained. First, it is possible that increased GSH is a result of oxidative stress. Because oxygen tension is higher in air than in venous blood (Lambertsen, 1974), parasites were exposed to higher concentrations of reactive oxygen intermediates during *in vitro* incubations than in the host (Fridovich, 1978). Thus, increased GSH levels might be required to protect the parasite against oxidative damage. This explanation seems inadequate, because the GSH increase was seen only in unpaired males, and oxidative stress would be expected whether the worms were paired or unpaired.

A second explanation is that like glucose (Cornford, 1986), GSH is provided to the female by the male *in copula*. If this were the case, then upon separation, transfer of GSH to the female would cease, and consequently, the level within the male would increase. Conversely, if the female were totally dependent on the male for GSH, then the level of GSH in unpaired females would be expected to fall during *in vitro* incubation, but it does not. Such an explanation is also inconsistent with the idea that GSH is not transported intact into cells, but rather is first degraded to amino acids, which are then transported to provide precursors for subsequent intracellular GSH biosynthesis (Abbott et al., 1984; Bannai and Tateishi, 1986).

When the female is absent from the gynecophoral canal, up to 50% more surface area of the male is exposed to the medium. Assuming that amino acid uptake is transtegumental (Asch and Read, 1975), GSH precursor transport might be increased, raising intracellular concentrations and as a consequence of mass action, the rate of GSH biosynthesis would increase. We think this is unlikely, because if increased parasite surface contact with the medium leads to increased GSH biosynthesis, then the GSH level of unpaired fe-



males would also be expected to rise. Although increased transport of L-glutamate has been observed in both separated males and females, compared with paired worms (Cornford, 1985), there is no change in the GSH content of unpaired females over 24 hr (Fig. 3).

Finally, it is conceivable that the increased GSH level observed is a marker of male worm metabolic activity that is influenced by the female. Nothing is known about the molecular events involved in the mating process. Altered metabolism might, for example, be required for attraction of the female. Control of this metabolic activity could result from transduction of tactile stimulation of receptors on the surface of the male or by female-to-male chemical signaling. Transfer of [ $^{14}\text{C}$ ] cholesterol from female to male schistosome has been suggested (Haseeb et al., 1985), providing a precedent for movement of chemicals from female to male. Although it is widely accepted that female schistosomes are dependent on males for a number of biological functions, effects of females on the physiology of males have not been extensively considered. Our observations support the hypothesis that chemical communication between the schistosome sexes is bidirectional.

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## THE MIDGUT HEMOLYSIN OF *IXODES DAMMINI* (ACARI: IXODIDAE)

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**ABSTRACT:** Midgut homogenates of the hard tick, *Ixodes dammini*, lyse erythrocytes from rabbits, rats, hamsters, and guinea pigs. The activity displays sigmoidal kinetics, has an alkaline pH optimum, and is activated by temperature. Hemolytic activity is lost when homogenates are incubated with trypsin or heated for 1 hr at 60 C. Activity is not detectable in nonfed ticks as well as ticks attached for up to 2 days to a host, but increases during the growth phase of feeding. Such activity is postulated to help the initial process of the blood meal digestion by releasing the contents of erythrocytes for further enzymatic hydrolysis.

Ixodid ticks stay attached for many days on their hosts while feeding. This feeding process can be divided into 2 phases, the growth phase and the rapid imbibition phase. Digestion and feeding proceed at a rapid rate during the growth phase. The expansion phase is comparatively brief, but most of the blood is taken during this period (Akov, 1982). Digestion by hard ticks is a slow intracellular process. Among the initial steps of blood meal digestion, histological observations indicate the presence of extracellular hemolysins involved in the lysis of erythrocytes (Hughes, 1954; Agdebe and Kemp, 1985).

Hemolysins have been described in the digestive tract of a variety of blood-sucking arthropods such as stable flies (Spates, 1981), triatomine bugs (Azambuja et al., 1983), tsetse flies (Gooding, 1977), and mosquitoes (Geering, 1975). The molecular mechanisms for inducing hemolysis are varied and include the combined action of proteinases and phospholipases (Geering, 1975), lipids (Spates, 1981), and polypeptides without apparent enzymic activity (Azambuja et al., 1983). Hemolysis of the blood meal in the midguts of soft ticks has also been demonstrated (Tatchell et al., 1973), but its nature was not characterized. Similarly, no hemolysins have been biochemically characterized from hard ticks.

To investigate whether the tick *Ixodes dammini* has a midgut hemolysin, experiments were carried out to determine the presence and characterize physicochemical behavior of such activity in adult female ticks.

### MATERIALS AND METHODS

All organic reagents were from Sigma (St. Louis, Missouri). Inorganic chemicals were obtained from

Fisher (Medford, Massachusetts). Distilled and deionized water were used.

Adult *Ixodes dammini* ticks were collected by flagging on Great Island (West Yarmouth, Cape Cod, Massachusetts) during the spring and fall of 1986. Ticks were stored at 4 C and at a relative humidity of 90% for 1-16 wk. They were then fed in groups of about 25 mating pairs in cloth bags taped to a rabbit's ear for 5 days. A collar was placed on each rabbit to prevent grooming.

Unless otherwise stated, at 4 days postattachment, ticks were removed by traction with forceps and dissected within 2 hr. Ticks were covered with phosphate-buffered saline (10 mM sodium phosphate, pH 7.4, 150 mM NaCl) and dissected inside a small petri dish. An incision on the dorsal cuticle exposed the diverticula, which were removed with forceps, washed into PBS, and transferred to a 0.4-ml conical plastic tube. Pools of 5 midguts were stored at -70 C until used, when they were homogenized in 2.5 ml of veronal-buffered saline (1.019 g/L Na-5,5 diethylbarbiturate, 8.3 g/L NaCl) in a glass homogenizer followed by centrifugation at 12,000 g for 5 min. For the experiment determining the time course of the hemolytic activity following tick attachment to a host, individual guts were dissected as above and each homogenized in 0.5 ml of buffer.

Pilocarpine was used to induce ticks to salivate (Tatchell, 1967; Ribeiro, 1987). Ticks were permitted to engorge for 4 days, and were then removed by traction. To collect saliva, 1  $\mu$ l of 5% (w/v) pilocarpine hydrochloride solution in methanol was applied topically to the dorsum of ticks restrained in glass slides by adhesive tape and held at 35 C in a humid chamber. Saliva was collected in glass tubes for 2 hr thereafter. In the following text the word saliva or tick saliva will mean pilocarpine-induced saliva of adult *I. dammini* female ticks.

Hemolytic tests were performed with washed rabbit red blood cells (RBC) collected into Alsever's solution, washed 4 times in phosphate-buffered saline (10 mM sodium phosphate, pH 7.4, 140 mM NaCl), and resuspended in veronal-buffered saline, pH 7.35, unless otherwise stated. For pH dependence assays of the hemolytic activity, citric acid-Tris buffer was used at a final concentration of 10 mM for each buffer. Final pH was adjusted with NaOH. Indicated volumes of tick midgut homogenates in veronal-buffered saline were added first, followed by erythrocytes. Final volume was 0.1 ml. Added erythrocytes were enough to give  $1 \pm$

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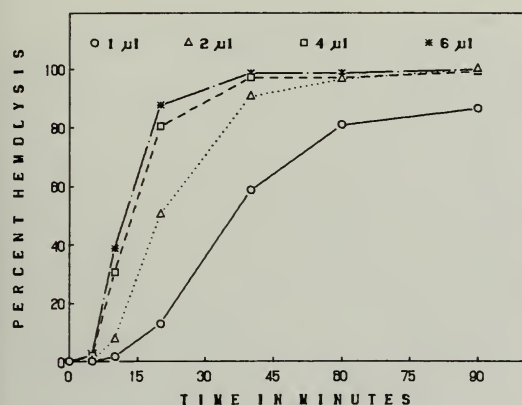


FIGURE 1. Time course of the hemolytic reaction induced by addition of different amounts of *Ixodes dammini* midgut homogenate. Experiment was done with a pool of 5 guts from adult female ticks fed for 4 days on a rabbit and homogenized in 2.5 ml of veronal-buffered saline. Indicated amounts of the homogenate supernatant of 12,000  $g \times 5$  min was added to a suspension of erythrocytes, with a total volume of 100  $\mu$ l. Symbols represent the average of a duplicate assay. Two other experiments using 1 and 5  $\mu$ l each of different gut preparations yielded essentially the same result.

0.05 absorption units at 405 nm after complete hypotonic lysis ( $\sim 5 \times 10^7$  cells/ml), in a final volume of 0.6 ml (light path = 1 cm). After incubation for indicated amounts of time at 37 C, 0.5 ml of 0.15 M NaCl was added to each tube, and tubes were immediately centrifuged (2,000  $g \times 4$  min). The optical density at 405 nm of the supernatant was measured on a spectrophotometer. Controls always included tubes with no added homogenate or no added red cells. Results are expressed as percent of cell lysis. To compare total amounts of hemolysin among different midgut preparations, 1 unit (U) of hemolytic activity was defined as the amount of material that hemolyzed 50% of the erythrocytes after 30 min and at 30 C (Smith et al., 1984). In practice, different amounts of the homogenate (1, 2, 4, 8, and 16  $\mu$ l) were added to the rabbit erythrocyte suspension and the value for 50% hemolysis obtained by interpolation. pH values larger than 8.5 were not tested when determining pH optimum for the hemolytic reaction due to excessive hemolysis in the control assays.

Trypsin treatment of the midgut homogenates was performed by adding 10  $\mu$ l of a solution of bovine trypsin at 2 mg/ml to 90  $\mu$ l of homogenate in veronal-saline and incubating for 30 min at 37 C. Controls were run without trypsin. Ethanol treatment of the homogenate was performed by adding 0.95 ml of cold ( $-25$  C) ethanol to 50  $\mu$ l of the homogenate. After incubation for 2 hr at  $-25$  C, the suspension was centrifuged at 10,000  $g$  for 5 min and the supernatant and precipitate dried separately under low pressure and reconstituted into 50  $\mu$ l of veronal-saline.

Protein was determined by the Coomassie blue method (Spector, 1981), using egg lysozyme as standard.

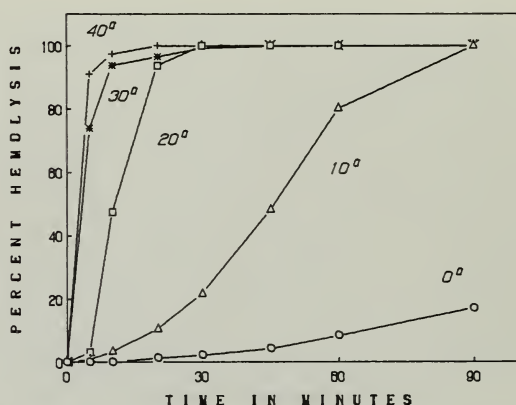


FIGURE 2. Temperature dependence of the hemolytic reaction induced by *Ixodes dammini* midgut homogenate. Five  $\mu$ l of homogenate were used per assay. Results are the average of a duplicate assay. Two other experiments using 0 and 30 C with 2 different midgut preparations yielded essentially the same result. Other conditions as in Figure 1.

## RESULTS

To investigate whether the midgut of *Ixodes dammini* ticks contained hemolytic activity, different amounts of midgut homogenate were incubated for different time intervals with a suspension of rabbit erythrocytes. Results indicate that the equivalent of 1/500 of 1 tick midgut induced more than 75% hemolysis within less than 1 hr, and that the degree of hemolysis was dependent on the concentration of midgut homogenates. Because the soluble protein concen-

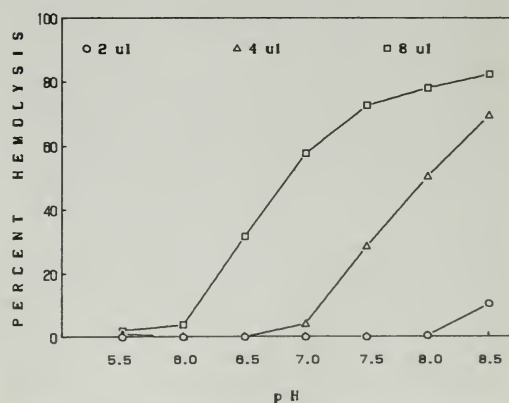


FIGURE 3. pH dependence of the hemolytic reaction induced by *Ixodes dammini* midgut homogenate. Citric acid-Tris (10 mM each) adjusted to the indicated pH was used as buffer. Three indicated concentrations of the midgut homogenate were used and the reaction was allowed to proceed for 20 min. Other conditions as in Figure 1.

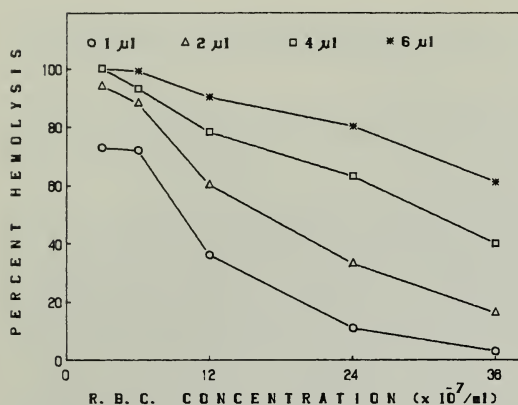


FIGURE 4. Erythrocyte dependence of the hemolytic reaction induced by *Ixodes dammini* midgut homogenate. Results are expressed as the percentage of cells that were lysed at each erythrocyte concentration. Reaction was allowed to proceed for 20 min. Two other experiments using 1 and 6  $\mu\text{l}$  of different gut homogenates provided essentially the same result.

tration in the homogenate used was 3.6 mg protein/ml, less than 4  $\mu\text{g}$  of protein was sufficient to produce measurable hemolytic activity. Notice the sigmoidal kinetics observed for all concentrations tested (Fig. 1).

Because feeding ticks imbibe fluids contaminated with their own saliva, there is the possibility that the hemolytic activity observed in midgut homogenates originated in the salivary glands. Accordingly, 10  $\mu\text{l}$  of tick saliva were incubated with 100  $\mu\text{l}$  of rabbit erythrocyte suspension for 1 hr at 37 C (5 different salivary samples were tested) but no hemolysis occurred. We conclude that the midgut hemolytic activity has a midgut origin.

The temperature dependence of the hemolytic activity was investigated at 0, 10, 20, 30, and 40 C. The hemolysis induced by tick midgut homogenates was activated by temperature (Fig. 2).

*Ixodes dammini* midgut hemolytic activity pH dependence was investigated by assaying different concentrations of the homogenate at a range of pH varying from 5.5 to 8.5. As shown in Figure 3, hemolytic activity proceeded faster at alkaline pH.

The dependence of the hemolytic activity on the erythrocyte concentration was investigated at different midgut homogenate concentrations. When 1  $\mu\text{l}$  of midgut homogenate was used, submaximal hemolysis occurred at the smaller concentration of RBC used during the incubation period (Fig. 4). At larger RBC concentrations, the fraction of cells hemolyzed decreased (Fig.

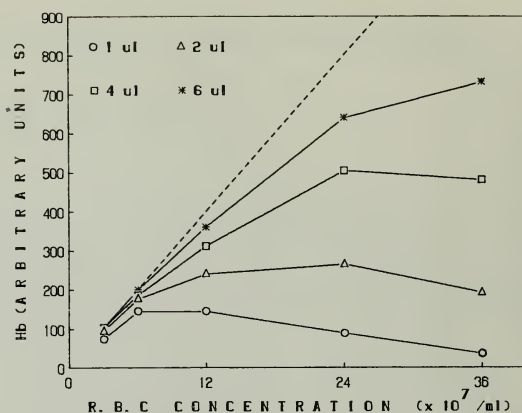


FIGURE 5. Erythrocyte dependence of the hemolytic reaction induced by *Ixodes dammini* midgut homogenates. Results are from the same experiment reported in Figure 4 but expressed as the absolute amount of hemoglobin released. For estimating hemoglobin release, a value of 100 was arbitrarily chosen as the total amount of 405-nm absorbing material released by hypotonic shock of  $3 \times 10^7$  erythrocytes. The dashed line represents 100% lysis.

4), as well as the total amount of hemoglobin released (Fig. 5), indicating that there was actually a reduced number of cells being lysed at larger RBC concentrations. At larger midgut concentrations, total hemolysis occurred at the smaller RBC concentrations (Fig. 4), and as the RBC concentration increased, there was an increase in the amount of hemoglobin released and thus an increase in the total number of cells lysed, although saturation is observed at larger RBC concentrations (Fig. 5).

The total soluble protein as well as total hemolytic activity of midguts obtained from ticks at different stages of feeding was measured. The hemolytic activity is expressed only after tick attachment to the host. No activity could be detected in ticks attached for less than 2 days, but by the third and fourth days the activity increased in parallel with the total soluble midgut protein (Fig. 6). Most (>75%) of the ticks were detached by the fifth day of feeding.

To investigate the specificity of the hemolytic activity toward erythrocytes derived from different species, midgut homogenates were incubated for different intervals of time with red blood cell suspensions from rabbit, rat, guinea pig, and hamsters. Hemolysis was observed with all erythrocytes tested, although some differences of the hemolysis rate were evident (Fig. 7).

To investigate the molecular nature of the

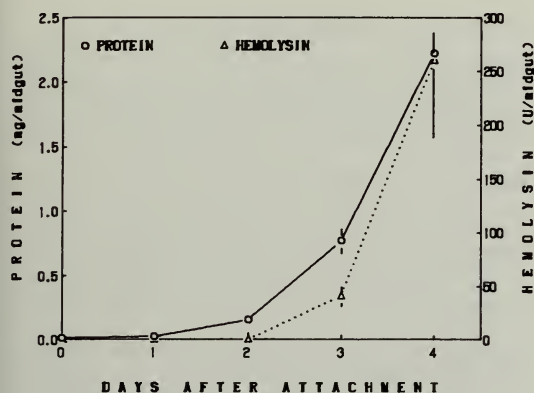


FIGURE 6. Soluble protein and hemolytic activity contained in supernatants of *Ixodes dammini* midgut homogenates derived from individual ticks with different times of attachment to rabbit hosts. Symbols and bars are the mean  $\pm$  SE of 5 different midgut homogenates. For more details, see Materials and Methods.

midgut hemolysin(s), midgut homogenates were submitted to several different treatments. Incubation of tick midgut homogenates with bovine trypsin for 30 min at 37 C destroyed the activity. Cold ( $-25$  C for 2 hr) ethanol precipitation of the homogenate (95% final ethanol) followed by vacuum drying of the resulting precipitate and supernatant also led to inactivation of the hemolytic activity. The activity was stable for several months if kept frozen ( $-25$  C), and suffered no loss of activity if kept for 1 day at 4 C. Complete inactivation occurred if kept at 22 C for 6 hr. Heat treatment of the homogenate (1 hr at 60 C) completely inactivated the hemolytic activity. The presence of 2 mM EDTA in the hemolysis assay did not change the activity, but the addition of 5 mM  $\text{CaCl}_2$  (in the absence of EDTA) led to reduction of the hemolysis from  $52 \pm 3\%$  to  $18 \pm 2\%$  hemolyzed (mean  $\pm$  SE,  $n = 4$ ) ( $5 \mu\text{l}$  of 1 tick midgut homogenate incubated for 20 min at 30 C).

## DISCUSSION

Blood-sucking invertebrates must lyse red cells to make hemoglobin available for proteolytic enzymes and further digestion. Histological evidence has indicated the presence of a hemolysin in the midgut of ixodid ticks (Hughes, 1954; Agdebe and Kemp, 1985). The presence of a midgut hemolysin in the tick *Ixodes dammini* is demonstrated in this paper and conditions for its assay determined. The following discussion will compare the characteristics of this hemolytic ac-

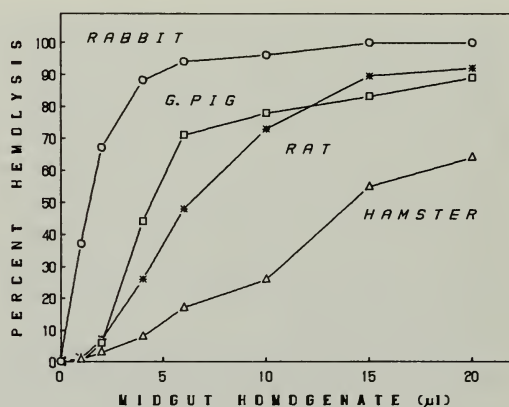


FIGURE 7. *Ixodes dammini* hemolytic activity on erythrocytes derived from different vertebrates. Indicated amount of homogenate was incubated with red cells from different vertebrates for 20 min. Symbols are the average of a duplicate experiment. Two other different midgut homogenates tested at 10  $\mu\text{l}$ /assay confirmed the results.

tivity to those from other sources and indicate its possible mode of action and physiological significance.

*In vitro* hemolysis of rabbit erythrocytes by *I. dammini* midgut homogenates displays sigmoidal kinetics (Fig. 1). This phenomenon is also observed for other lysins, such as those derived from *Schistosoma mansoni* worms (Kasschau and Dresden, 1986) and the amoeba *Naegleria fowleri* (Lowery and McLaughlin, 1984). This may indicate the presence of a multicomponent lytic system, as found for *Naegleria* (Lowery and McLaughlin, 1984). Single-hit systems yield a fixed number of lysed cells at large cell-to-hemolysin ratios and are not inhibited by excess cells (Jorgensen et al., 1980). The actual decrease in the number of cells lysed when the erythrocyte concentration was raised, noticeable at smaller homogenate concentrations, also supports a multicomponent system.

In contrast to the *Schistosoma* hemolysin (Kasschau and Dresden, 1986), which is more active at acidic pH, *I. dammini* hemolysin is more active at alkaline pH. The pH of the midgut of ixodid ticks during feeding is not known (Akov, 1982).

Although the nature of *I. dammini* hemolytic activity cannot be precisely defined, several possibilities can be excluded. Phospholipase  $A_2$  has hemolytic activity, but such enzymes require calcium (Hanahan, 1971). *Ixodes dammini* hemolytic activity was inhibited by calcium and is not



modified by the presence of EDTA. These results suggest that phospholipase A<sub>2</sub> was not involved in the hemolytic reaction. Lipids are involved with the midgut hemolytic activity of the stable fly *Stomoxys calcitrans* (Spates, 1981). *Ixodes dammini* hemolysin activity does not seem to be of lipidic nature due to its destruction by trypsin, nonrecovery from ethanolic extracts, and temperature sensitivity. The sensitivity to trypsin and heat indicates the mediation of peptides in the hemolytic activity.

It appears that the gut hemolysin has a gut origin and its presence is stimulated by the blood meal because no activity can be measured in tick guts up to the second day postattachment to a host (Fig. 6). The possibility that *I. dammini* gut hemolysin could be of salivary origin, being reingested during the blood meal, was discarded because saliva of this same tick in the same hemolytic assay did not induce hemolysis (even at 10  $\mu$ l of saliva/100  $\mu$ l assay). In fact, saliva prevented rabbit erythrocyte hemolysis by the human alternative complement pathway, by preventing C3 fixation to activating surfaces (Ribeiro, 1987). This is in accordance with Balashov's (1968) suggestion of a gut origin for tick midgut hemolysins.

*Ixodes dammini* midgut homogenates induced hemolysis in red cells originating from different species. Rabbit erythrocytes were most sensitive, as was the case for the hemolymph hemolytic activity of *Mercenaria mercenaria* (Anderson, 1981). The reason for this greater sensitivity is unknown, and can only be speculated. Possible explanations are a larger affinity of the hemolysin for surface receptors on rabbit erythrocytes (Howard and Buckley, 1982), or increased susceptibility of rabbit erythrocytes for the hemolysis mechanism. Whatever the reason, the species differences may bear little physiological importance for the tick's digestive process unless the rate of hemolysis becomes a limiting step in the tick digestive process. The relevant fact is the ability of this hemolytic activity to enable proteolytic attack of the erythrocyte contents.

#### ACKNOWLEDGMENTS

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## SECRETORY PROTEIN BIOSYNTHESIS IN SNAIL HEMOCYTES: IN VITRO MODULATION BY LARVAL SCHISTOSOME EXCRETORY-SECRETORY PRODUCTS

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**ABSTRACT:** Circulating hemocytes of the snail, *Biomphalaria glabrata*, synthesize and secrete a variety of polypeptides when maintained *in vitro* in serum-free medium containing [ $^{35}$ S] methionine. SDS-PAGE/fluorographic analysis of supernatants from resistant snail (10-R2-OK strain) hemocyte cultures revealed the presence of numerous labeled polypeptides ranging in M<sub>r</sub> from 220 to 14 kDa. Most of these same proteins were also produced by hemocytes of a susceptible *B. glabrata* strain (M-line), but the overall rate of secretory protein synthesis was reduced from that of resistant snail cells. In addition, excretory-secretory (ES) products contained in supernatants from *Schistosoma mansoni* miracidial transformation and 1-day primary sporocyst cultures stimulated increases in the synthesis of various polypeptides. Particularly striking was a 3-fold increase in the synthesis of a 66-kDa secretory polypeptide by hemocytes of both snail strains, and a concomitant increase in M-line hemocytes and decrease in 10-R2-OK cells of a 63-kDa polypeptide. Overall, however, the level of ES product-induced secretory protein synthesis was greater in 10-R2-OK snail hemocytes than in those of the M-line strain. Exposure of a nonhemocytic *B. glabrata* cell line to parasite culture supernatants had no stimulatory/inhibitory effect on labeled protein output, suggesting that the observed hemocyte response may be snail cell-type specific. Finally, the larval ES components responsible for modulating hemocyte protein metabolism are mainly concentrated in a heat-stable fraction composed of molecules of > 30 kDa. However, the loss of the ability of heated parasite products to stimulate synthesis of certain hemocyte proteins and the presence of minor stimulating activity in a low molecular weight fraction (< 10 kDa) implies the possible existence of multiple larval components affecting formation of specific hemocyte secretory polypeptides. It is concluded that snail hemocytes are capable of *in vitro* synthesis and secretion of a variety of methionine-containing polypeptides, and that ES products of early larval schistosomes can modulate (i.e., stimulate or inhibit) this metabolic process. A differential response of susceptible vs. resistant hemocytes to larval products suggests that the degree to which these cells can be metabolically activated may determine their cytotoxic effectiveness.

The cellular basis for innate resistance in the snail, *Biomphalaria glabrata*, to larval schistosome infection appears to lie in the ability of circulating host blood cells (hemocytes) to recognize and encapsulate the early developing parasite stages (Bayne, 1983). This direct hemocyte involvement in antiparasite reactions has been confirmed in *in vitro* cytotoxicity assays in which hemocytes from resistant, but not susceptible, snails were shown to mediate larval schistosome killing (Bayne et al., 1980a, 1980b). One hypothesis suggested by these authors to explain the differential hemocyte cytotoxic response is that resistant snail cells occur in a naturally activated state, relative to those of susceptible hosts, which makes them more effective in killing reactions. Such a hypothesis is supported by the finding that hemocytes from a resistant *B. glabrata* strain (10-R2) possess a greater distribu-

tion and abundance of various lysosomal enzymes than cells of the susceptible M-line strain (Granath and Yoshino, 1983a). In addition, the induction of changes in hemocyte hydrolytic enzyme activities by parasite infection (Cheng et al., 1978; Granath and Yoshino, 1983b) or in *in vitro* cytotoxic potential by exposure of hemocytes to soluble plasma factors (Bayne et al., 1980b) also suggests that the state of hemocyte activation may play a crucial role in determining host susceptibility to infection.

In the present study, the metabolic activity of hemocytes from a schistosome-resistant and susceptible strain of *B. glabrata* was compared using *in vitro* biosynthesis of secretory proteins as a qualitative and quantitative indicator of their state of cellular activation. Thus, [ $^{35}$ S] methionine-labeled secretory proteins of cultivated hemocytes were analyzed using SDS-PAGE and fluorography to (1) determine whether cells from these defined snail strains differed in their levels of protein synthesis or secretory protein output and (2) determine whether larval schistosome excretory-secretory (ES) products could influence *in vitro* hemocyte protein synthetic activity.

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## MATERIALS AND METHODS

### Preparation of snail hemocytes

*Schistosoma mansoni*-resistant (10-R2-OK) and susceptible (M-line) strains of *B. glabrata* were used in this study. The resistant *B. glabrata* strain, designated 10-R2 by Richards (1975), has been renamed 10-R2-OK (10-R2-Oklahoma strain) and denotes a specific snail subcolony, which over the last 10 yr of laboratory cultivation may or may not have retained genetic identity with the original snail stock. The average infection rate for 10-R2-OK, using miracidia of the NMRI strain of *S. mansoni*, is approximately 12%. Details of snail colony maintenance are given elsewhere (Yoshino, 1981).

Cultivation of hemocytes for *in vitro* metabolic studies was accomplished as follows: The external surface of snails (13–15 mm in shell diameter) was first swabbed with 70% ethanol, allowed to dry, and then the snails were placed into a sterile petri dish containing sterile distilled water (dH<sub>2</sub>O) with a 1:2 dilution of 1 × pen-strep solution (Sigma Chemical Co., St. Louis, Missouri). The snails were allowed to soak for 30 min and then rinsed twice with sterile dH<sub>2</sub>O and soaked for an additional 30 min. In a culture hood, snails were removed from dishes, blotted dry, and hemolymph was extracted from the headfoot (Sminia and Barendsen, 1980). Hemolymph from several snails was initially pooled in a sterile petri dish for about 3 min to allow for settling of noncellular debris. Aliquots (50- $\mu$ l) were then distributed to the desired number of wells of a 96-well culture plate (Costar, Cambridge, Massachusetts). Typically, a single well contained hemocytes from 150 to 200  $\mu$ l of 10-R2-OK hemolymph, whereas a comparable number of M-line cells required the loading of about 350  $\mu$ l of hemolymph. Hemocytes were allowed to settle and adhere to well bottoms for 2 hr, after which time the plasma and nonadherent cells were removed by washing monolayers 4 times with serum-free *Biomphalaria glabrata* embryo culture medium containing 100  $\mu$ g gentamicin/ml (incomplete or I-Bge medium; Hansen, 1976). A final volume of 60  $\mu$ l I-Bge was then added to each culture. It was estimated that adherent hemocytes comprised approximately 90–95% of the total cell population.

### Metabolic labeling experiments

Synthesis of secretory and nonsecretory proteins by cultured hemocytes was assessed by incorporation of [<sup>35</sup>S] methionine ([<sup>35</sup>S] met; specific activity >800 Ci/mmol; NEN, Boston, Massachusetts). Metabolic labeling was initiated by the introduction of 60  $\mu$ l of I-Bge containing 55  $\mu$ Ci of [<sup>35</sup>S] met to freshly prepared hemocyte cultures. These control cultures (60  $\mu$ l I-Bge + 60  $\mu$ l [<sup>35</sup>S] met I-Bge) were compared to parasite test cultures in which hemocytes were incubated in the presence of larval ES products (60  $\mu$ l of transformation or sporocyst culture medium + 60  $\mu$ l [<sup>35</sup>S] met I-Bge) (see preparation of ES products below). Hemocyte cultures were maintained at 26 C and >70% humidity for 48–52 hr. The number of cultured hemocytes was estimated for each well at the beginning of the cultivation period by counting the cells contained in 4 random microscopic fields at 200× magnification. Hemacytometer counts of hemocytes in the initial hemolymph pools were not used because not all cells (e.g., non-

adherent hemocytes) would be represented in final culture wells following monolayer washes. High viability (>90%), based on cell morphology and the ability of cultured hemocytes to remain attached to the substrate, was maintained during the cultivation period. Microbial contamination was monitored by periodic microscopic examination of cultures.

Following incubation, hemocyte culture supernatants were collected, centrifuged at 11,000 g to remove cellular debris, and mixed with sample buffer (Laemmli, 1970; 2 parts supernatant: 1 part buffer) containing 2 mM phenylmethylsulfonylfluoride (PMSF). Hemocytes were washed once with fresh I-Bge and solubilized in PMSF-containing sample buffer. Detached hemocytes removed during the initial isolation of culture supernatants or the washing step were “added back” to their respective cell sample by treating centrifuged pellets with their corresponding sample buffer. Both cell and supernatant preparations were adjusted by volume to accommodate the different number of hemocytes in each culture well. This standardization procedure permitted a direct comparison of hemocyte proteins between snail strains and between parasite-treated and untreated cultures within a single experimental replicate (i.e., under identical culture conditions). Experiments were replicated from 3 to 7 times on different groups of snail hemocytes.

### Preparation of larval schistosome excretory-secretory (ES) products

Schistosome miracidia (NMRI strain) were hatched from eggs axenically recovered from 8-wk-infected mouse livers (Yoshino et al., 1977) and subsequently placed into Bge medium (Hansen, 1976) supplemented with 30% heat-inactivated fetal bovine serum and gentamicin (100  $\mu$ g/ml medium). Miracidia placed in this medium transformed to primary sporocysts within about 6 hr of cultivation at 26 C. Sporocysts were subsequently washed free of shed ciliated epidermal plates, and sporocysts and isolated plates were cultured separately in wells of a 24-well culture plate containing approximately 1 ml of I-Bge medium. Culture supernatants were collected daily for a period of 6 to 8 days with fresh I-Bge being used as replacement medium. Thus, larval products secreted at different days of *in vitro* development were available for testing. Supernatants were centrifuged, filter-sterilized, and stored at –10 C before use (usually within a week). Sporocyst cultures were routinely monitored for microbial contamination for 8–10 days posttransformation to assure sterility of harvested larval media.

Miracidia also were transformed directly in I-Bge (the majority of parasites usually transformed to sporocysts within 24 hr), and the culture supernatant, designated transformation medium, was used in metabolic labeling experiments designed to test the effects of early larval ES products on host hemocyte protein synthesis and secretion. In these experiments, either whole, unfractionated transformation medium, or fractionated medium was employed in the metabolic labeling assay described above. The latter medium consisted of 2 molecular fractions (<10 kDa and >30 kDa) that were separated by membrane ultrafiltration (Amicon Corp., Danvers, Massachusetts). In addition, the effect of heating larval ES products on their ability to modulate

synthesis of hemocyte secretory proteins was examined by exposing 10-R2-OK cell cultures to 60  $\mu$ l of transformation medium that had been heated to 100 C for 6 min. Hemocyte cultures receiving 60  $\mu$ l of I-Bge or 60  $\mu$ l of untreated transformation medium served as negative and positive stimulation controls, respectively.

Finally, the secretory proteins from a nonhemocytic *B. glabrata* embryonic cell line (Bge, ATCC# CRL 1494) were assessed in parallel experiments to determine whether any effect of schistosome ES products on hemocyte protein synthesis was snail cell-type specific. In this experiment, cultures of Bge cells were exposed to larval transformation supernatants in the presence of [ $^{35}$ S] met (55  $\mu$ Ci/well) as previously described for hemocytes. Bge cells cultivated in the absence of larval supernatant served as nonstimulated controls. Bge culture supernatants were harvested after 50 hr, adjusted for cell number, and analyzed by SDS-PAGE/fluorography.

#### Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography

SDS-PAGE was carried out in 10% slab gels at 30 mA according to a procedure modified from Laemmli (1970) as described by Boswell et al. (1987). Following electrophoresis, the molecular weight marker lanes were cut from the slab gels, fixed in 50% methanol, stained with ammoniacal silver (Wray et al., 1981), and photographed. The remainder of each gel was fixed in 50% methanol and processed for fluorography using a DMSO-PPO impregnation method (Bonner and Laskey, 1974). X-ray films (Kodak X-AR5) were exposed to gels for 3–7 days at –80 C to produce autoradiograms. Fluorograms were routinely scanned using either a soft laser densitometer (LKB Model 2202, Bromma) or a scanning spectrophotometer (Beckman DU-8B) at a wavelength of 500 nm. The resulting densitometric readings were used to quantify the relative levels of secretory polypeptide synthesis by hemocytes of the 2 snail strains.

#### Quantitative analysis

Although the electrophoretic patterns of hemocyte-synthesized proteins were quite consistent within a single replicate and from replicate to replicate, the quantitative output varied considerably between replicates, even when standardized to equal cell numbers. The reasons for this trial-to-trial variation are unclear; however, because experiments were performed over the course of a year, hemocyte sensitivity to different batches of media, different lots of [ $^{35}$ S] met, or changes associated with aging media, the use of different groups of parasite-conditioned supernatants, or possible fluctuations in endogenous seasonal or reproductive cycles in stock snails may be among contributing factors. Therefore, in order to compare quantitatively the relative rates of secretory protein synthesis between and within groups of hemocytes of the M-line and 10-R2-OK strains, we chose to standardize protein output by representing the labeled protein contribution for each hemocyte group as a proportion of the total synthesized protein of the 2 groups being compared within a single experimental replicate.

We accomplished this calculation by, first, deter-

mining the areas under densitometric scans of fluorographs for each hemocyte culture replicate (M-line control, 10-R2-OK control, M-line parasite-stimulated, and 10-R2-OK parasite-stimulated). Then, within each replicate, paired comparisons were made by calculating the proportion of labeled proteins contained in one lane relative to those contained in a separate lane representing a different treatment group. As an example, a comparison between the total secretory proteins synthesized by M-line and 10-R2-OK control hemocytes was calculated as follows:

$$\frac{\text{area (M-line control)}}{\text{area (M-line + area (10-R2-OK control))}} = \frac{\text{proportion of total synthesized protein contributed by M-line hemocytes}}{\text{proportion of total synthesized protein contributed by 10-R2-OK hemocytes}}$$

$$\frac{\text{area (10-R2-OK control)}}{\text{area (M-line + area (10-R2-OK control))}} = \frac{\text{proportion of total synthesized protein contributed by 10-R2-OK hemocytes}}{\text{proportion of total synthesized protein contributed by M-line hemocytes}}$$

In these paired comparisons, it was assumed that if the 2 groups being compared (e.g., M-line vs. 10-R2-OK controls) did not differ from each other, the proportion of synthesized protein contributed by each group to the total would be 0.5 or 50%. If, on the other hand, one group was producing more material relative to the other, the proportion of labeled protein contributed to the total would increase for the more productive group. Using this method, within each replicate, we compared the total secretory protein synthesized for M-line control vs. 10-R2-OK control, M-line stimulated vs. 10-R2-OK stimulated, M-line control vs. M-line stimulated, and 10-R2-OK control vs. 10-R2-OK stimulated. Identical sets of data collected for each experimental replicate were then pooled to determine the average proportion of labeled proteins contributed by the 2 snail strains for between-strain comparisons, or under control and parasite-stimulated conditions to determine within-strain comparisons. Statistical differences in the proportion of radiolabeled polypeptides produced by the different control/treatment groups were determined using 1-way analysis of variance (ANOVA) of arcsine-transformed data.

A similar quantitative technique was used to assess whether larval-conditioned medium was stimulating synthesis of individual hemocyte secretory polypeptides in M-line and 10-R2-OK cell cultures. In this analysis, the areas of single fluorographed bands representing a variety of  $M_r$  were determined and the relative proportion of a given polypeptide synthesized under control and parasite-stimulated conditions was calculated for each experimental trial. Arcsine-transformed proportional data for each band were pooled from replicate trials and analyzed by ANOVA.

#### RESULTS

*In vitro*-cultivated hemocytes from *B. glabrata* synthesize and secrete a variety of polypeptides



TABLE I. Quantitative comparison of total secretory proteins synthesized by *Biomphalaria glabrata* hemocytes (M-line and 10-R2-OK strains) under parasite-stimulated (stim) and unstimulated (control) conditions. Stimulation of hemocyte cultures was accomplished by in vitro exposure of cells to larval transformation medium or 8-day sporocyst supernatant (8-day sup).

Groups compared	Proportion of total synthesized protein	ANOVA	% Increase‡
M-line control vs. 10-R2-OK control	0.399 ± 0.02*	$F[1,12] = 72.25; P < 0.001, n = 7$	50.6
M-line control vs. M-line stim	0.384 ± 0.03	$F[1,4] = 26.64; P < 0.01, n = 3$	60.4
10-R2-OK control vs. 10-R2-OK stim	0.616 ± 0.04	$F[1,10] = 36.56; P < 0.001, n = 6$	42.1
10-R2-OK control vs. 10-R2-OK stim	0.413 ± 0.03	$F[1,4] = 0.410; \text{NS}^\dagger, n = 3$	7.9
10-R2-OK stim (8-day sup) vs. M-line stim	0.519 ± 0.04	$F[1,4] = 24.40; P < 0.01, n = 3$	42.7
10-R2-OK stim vs. 10-R2-OK stim	0.412 ± 0.02		
10-R2-OK stim	0.588 ± 0.03		

\* Average proportion ± 1 SEM.

† Not significant.

$$\ddagger \% \text{ Increase of 10-R2-OK over M-line hemocytes (M-line vs. 10-R2-OK)} = \frac{\text{avg. proportion 10-R2-OK protein} - \text{avg. proportion M-line protein}}{\text{avg. proportion M-line protein}} \times 100.$$

$$\% \text{ Increase stimulated over control hemocytes (control vs. stim)} = \frac{\text{avg. proportion stimulated protein} - \text{avg. proportion control protein}}{\text{avg. proportion control protein}} \times 100.$$

ranging in  $M_r$  from 14 to 220 kDa (Fig. 1). Although the patterns of secretory proteins were similar for hemocytes of both the M-line and 10-R2-OK snail strains (Fig. 1, lanes A and C, respectively), the basal (control) output of total labeled polypeptides from 10-R2-OK snail cells was elevated in comparison to M-line hemocytes (Table I). The occurrence of a few secretory components, especially the 19-kDa band, was highly variable in supernatants of M-line hemocytes, but concentrations were consistently lower than corresponding polypeptides from resistant cell cultures. Synthesis of methionine-containing cellular proteins (i.e., those in association with whole, intact hemocytes) was similar, both qualitatively and quantitatively, for M-line and 10-R2-OK snail hemocytes (Fig. 1, lanes A' and C', respectively).

Exposure of hemocytes *in vitro* to supernatants from cultured *S. mansoni* larvae resulted in an augmentation of various [ $^{35}\text{S}$ ] met-labeled polypeptides released from M-line and 10-R2-OK hemocytes (Fig. 1, lanes B and D; Table I). Parasite-induced increases in labeled secretory polypeptides of  $M_r$  220, 66, 63, 50, 46, 36, and 22 kDa were noted for M-line hemocytes, whereas 10-R2-OK cells exhibited increased output of

polypeptides of 180, 163, and 19 kDa, in addition to most of those observed to increase in M-line hemocyte cultures (Table II). Particularly striking was a 3-fold increase in the apparent synthesis and release of a 66-kDa polypeptide in hemocytes of both snail strains (Fig. 1, Table II), and the induction in 10-R2-OK hemocytes of new polypeptides of  $M_r$  156 and 105 kDa (Figs. 1–3). The 63-kDa polypeptide, which increased in concentration in parasite-stimulated M-line hemocyte cultures, typically decreased in similarly treated 10-R2-OK cultures (Table II). It should be noted that several of the polypeptide “bands” designated by a specific  $M_r$  (e.g., 220, 50, and 36 kDa) were actually composed of multiple components. However, these multiple bands were too closely grouped for effective quantitative resolution by scanning densitometry, and, therefore, were treated in this analysis by single  $M_r$  identifications.

Culture supernatants from transforming miracidia and isolated 1-day cultured primary sporocysts exerted a similar modulating effect on hemocyte protein synthetic/secretory activity. However, ES products from cultured 8-day sporocysts failed to stimulate either quantitative or qualitative changes in hemocyte protein secre-



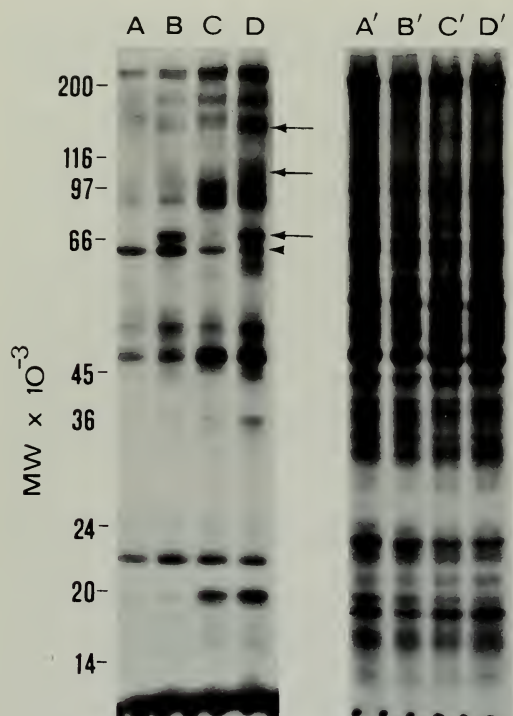


FIGURE 1. Fluorograms of [<sup>35</sup>S] methionine-labeled polypeptides appearing in hemocyte culture supernatants (A–D) and whole hemocyte extracts (A'–D'). In each replicate, supernatant or extract loads for each lane were standardized according to the number of hemocytes contained in each culture well. Molecular weight markers are indicated along the left margin. Lanes A and B represent examples of labeled M-line *B. glabrata* hemocyte secretory polypeptides from control and schistosome ES product-exposed hemocyte cultures, respectively. Lanes C and D represent the secretory polypeptides synthesized by 10-R2-OK snail hemocytes under control and ES product-exposed conditions, respectively. Arrows denote, in order of descending molecular weights, examples of parasite-induced hemocyte polypeptides of M<sub>r</sub> 156, 105, and 66 kDa. Arrowhead indicates a 63-kDa component. Lanes A'–D' are fluorograms of solubilized whole hemocytes from the cultures represented in lanes A–D, respectively.

tory activity (Table I; Fig. 2, lane D). In addition, examination of the effects of high (> 30 kDa) and low (< 10 kDa) molecular weight fractions of larval culture supernatants on 10-R2-OK hemocyte secretory protein synthesis revealed that parasite components of > 30 kDa were responsible for most of the hemocyte-stimulating activity (Fig. 2, lane B). This was most evident in the induction/augmentation of polypeptides of M<sub>r</sub> 156, 105, 66, and 50 kDa. Hemocyte-stimulating activity was also detected in the < 10-kDa fraction,

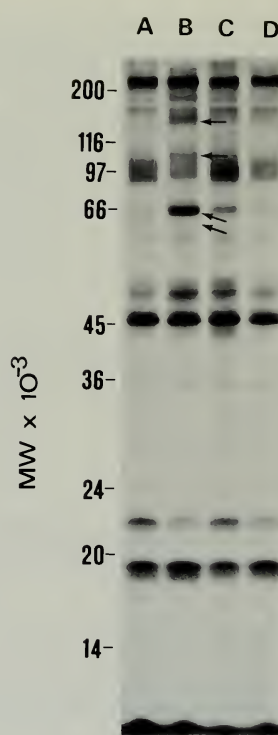


FIGURE 2. Effects of high (> 30 kDa) and low (< 10 kDa) molecular weight (MW) *S. mansoni* ES products (from transformation medium), and culture products of 8-day cultivated sporocysts on 10-R2-OK hemocyte secretory protein synthesis. Fluorograms represent SDS-PAGE-separated supernatants from control (lane A), high MW fraction-exposed (lane B), low MW fraction-exposed (lane C), and 8-day sporocyst-exposed (lane D) hemocyte cultures. Note that larval-induced synthesis of the hemocyte 156-, 105-, and 66-kDa polypeptides and modulation of the 63-kDa peptide (lane B, arrows) reside principally in the high MW parasite ES fraction.

most notably affecting synthesis of hemocyte polypeptides of M<sub>r</sub> 66 and 50 kDa, and a group in the 92–100-kDa range (Fig. 2, lane C). Heat treatment of parasite culture supernatants had little effect on the ability of larval ES products to stimulate synthesis of certain hemocyte secretory polypeptides (e.g., 66 and 50 kDa), whereas enhanced synthesis of other proteins (e.g., 180 and 19 kDa) was abrogated (Fig. 3).

Finally, in contrast to its ability to stimulate hemocyte secretory protein metabolism, larval schistosome culture products had no effect on secretory protein synthesis in snail Bge cells, a nonhemocytic *B. glabrata* cell line. As shown in Figure 4, output of labeled secretory polypep-

TABLE II. Quantitative comparison of individual polypeptides synthesized by *Biomphalaria glabrata* hemocytes under parasite-stimulated (stim) and unstimulated (control) conditions. Hemocyte cultures were stimulated by in vitro exposure to larval transformation medium throughout the incubation period. The percentage (%) increase/decrease was calculated as in Table I (control vs. stim).

Protein band (kDa)	Snail strain (n)	Avg. proportion protein synthesized under control conditions	Avg. proportion protein synthesized under stim conditions	ANOVA (control vs. stim)	% Increase/decrease
220	M-line (3)	0.430 ± 0.015*	0.570 ± 0.015	$F = 41.63$ $P < 0.01$	+32
	10-R2-OK (5)	0.430 ± 0.035	0.570 ± 0.035	$F = 8.12$ $P < 0.05$	+32
180	M-line	0.477 ± 0.024	0.523 ± 0.025	$F = 1.88$ NS†	—
	10-R2-OK	0.384 ± 0.032	0.616 ± 0.032	$F = 38.41$ $P < 0.01$	+63
163	M-line	0.523 ± 0.018	0.477 ± 0.018	$F = 3.50$ NS	—
	10-R2-OK	0.382 ± 0.024	0.618 ± 0.024	$F = 45.71$ $P < 0.01$	+62
100	M-line	0.463 ± 0.028	0.537 ± 0.028	$F = 3.32$ NS	—
	10-R2-OK	0.486 ± 0.025	0.514 ± 0.025	$F = 0.61$ NS	—
92	M-line	0.483 ± 0.038	0.517 ± 0.038	$F = 0.39$ NS	—
	10-R2-OK	0.490 ± 0.025	0.510 ± 0.025	$F = 0.33$ NS	—
66	M-line	0.200 ± 0.025	0.800 ± 0.025	$F = 199.84$ $P < 0.001$	+300
	10-R2-OK	0.210 ± 0.015	0.790 ± 0.015	$F = 524.56$ $P < 0.001$	+276
63	M-line	0.443 ± 0.012	0.557 ± 0.012	$F = 44.02$ $P < 0.01$	+25.7
	10-R2-OK	0.550 ± 0.030	0.450 ± 0.030	$F = 5.44$ $P < 0.05$	-18.2
50	M-line	0.380 ± 0.035	0.620 ± 0.035	$F = 22.92$ $P < 0.02$	+63.2
	10-R2-OK	0.376 ± 0.023	0.624 ± 0.023	$F = 57.75$ $P < 0.002$	+66
46	M-line	0.413 ± 0.020	0.587 ± 0.020	$F = 35.92$ $P < 0.01$	+42.1
	10-R2-OK	0.440 ± 0.019	0.560 ± 0.019	$F = 18.92$ $P < 0.01$	+27.3
36	M-line	0.417 ± 0.037	0.583 ± 0.037	$F = 10.05$ $P < 0.05$	+39.8
	10-R2-OK	0.354 ± 0.014	0.646 ± 0.014	$F = 216.60$ $P < 0.001$	+82.4
22	M-line	0.437 ± 0.010	0.563 ± 0.010	$F = 178.89$ $P < 0.001$	+28.8
	10-R2-OK	0.526 ± 0.050	0.474 ± 0.050	$F = 0.56$ NS	—
19	M-line	0.467 ± 0.064	0.533 ± 0.064	$F = 0.549$ NS	—
	10-R2-OK	0.442 ± 0.029	0.558 ± 0.029	$F = 8.28$ $P < 0.05$	+26.2

\* Average proportion ± 1 SEM.

† Not significant.

tides from Bge cells was identical in parasite-treated and untreated cultures.

## DISCUSSION

Even though exposure of a snail host to a trematode miracidium can result in only 1 of 2 out-

comes (infection or no infection), the degree of natural host susceptibility to their larval trematodes appears to be quantitative in nature (Lic, 1982). In other words, the determination of susceptibility is represented as a continuum in which successful infection of a snail with a given larval

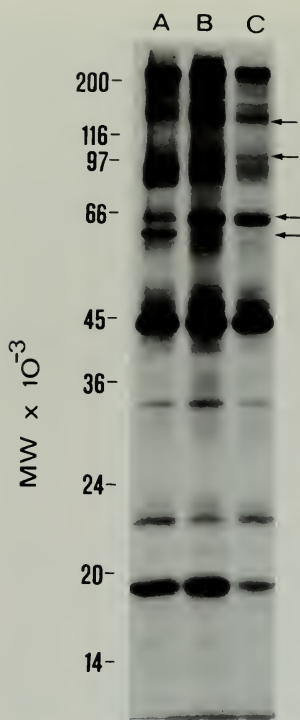


FIGURE 3. Effect of heat treatment (100 C, 6 min) of *S. mansoni* culture supernatants on the ability of parasite ES products to stimulate *in vitro* secretory protein synthesis in 10-R2-OK snail hemocytes. Fluorographs of hemocyte secretory products synthesized under control (unexposed), larval supernatant-exposed (nonheated), and heat-treated, larval supernatant-exposed conditions are depicted in lanes A, B, and C, respectively. Arrows indicate the 156-, 105-, 66-, and 63-kDa polypeptides.

exposure is dependent upon the level of innate resistance (or susceptibility) factors present within the host and the inherent infectivity of the parasite (Basch, 1975). This conclusion is based on numerous laboratory infection studies (Basch, 1976), which show that susceptibility of a snail population is rarely 100%, indicating that within a population, some hosts possess quantitatively higher or lower levels of innate resistance than others to challenge with a specific larval strain. More recent experiments have demonstrated that the level of resistance in normally susceptible *B. glabrata* snails to defined *S. mansoni* strains can be "boosted" or heightened by exposing snail hemocytes *in vitro* or *in vivo* to plasma of resistant hosts (Bayne et al., 1980b; Loker and Bayne, 1982; Granath and Yoshino, 1984). These results support the notion that the quantity (concentration) of yet unidentified host factor(s) may play

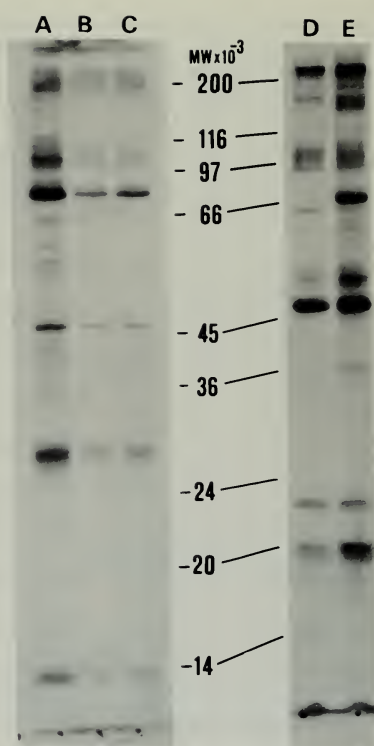


FIGURE 4. Effect of larval *S. mansoni* culture ES products on protein synthesis in the *B. glabrata* embryonic (Bge) cell line. Fluorograms of Bge secretory polypeptides from parasite-exposed (lanes A and B) and unexposed control (lane C) cultures. Sample loads for lanes B and C were equilibrated to cell number before SDS-PAGE separation. Fluorograms of 10-R2-OK snail hemocyte secretory polypeptides from unexposed control (lane D) and parasite-exposed test (lane E) hemocyte cultures are included for comparison purposes.

an important role in determining whether or not infection will result from a parasite-host encounter.

To explain why hemocytes from resistant *B. glabrata* strains (13-16-R1, 10-R2) are more effective than those of susceptible hosts (M-line strain) in the *in vitro* killing of larval *S. mansoni*, Bayne et al. (1980b) have suggested that resistant snail hemocytes may normally occur in a state of cytotoxic activation, whereas cells from susceptible strains do not. The observation that hemocytes from snails of the 10-R2 strain naturally possess a greater distribution and abundance of lysosomal hydrolases than cells from the susceptible M-line *B. glabrata* strain lends support to the "activated hemocyte" hypothesis (Granath and Yoshino, 1983a). In the present



study, using *in vitro* synthesis of secretory polypeptides as a measure of cellular metabolism, we now have evidence consistent with the notion that resistant 10-R2-OK hemocytes are metabolically more active than their susceptible snail counterparts. The finding that whole solubilized hemocyte preparations from the 2 snail strains did not appreciably differ from each other implies that the greater accumulation of labeled secretory proteins in 10-R2-OK culture supernatants, when compared to similarly treated M-line cultures, was due to a higher rate of polypeptide synthesis and secretion by resistant cells. It still remains an unresolved question whether this apparent difference in metabolic activity between susceptible and resistant snail hemocytes is responsible for their differential ability to destroy schistosome sporocysts *in vitro*. However, we chose to analyze the secretory proteins from snail hemocytes, not only for their convenience as markers for measuring *in vitro* basal metabolism, but also as a first step in analyzing the specific proteins elaborated by these effector cells in response to parasite stimulation. It is assumed that at least some of these products are involved either directly in parasite killing or indirectly, by serving in the regulation of other hemocyte defense-related activities.

Both stimulating and suppressing effects of larval trematodes on snail blood cell activity have been described. For example, natural infections have been shown to stimulate a rapid leukocytosis (Stumpf and Gilbertson, 1978, 1980; Granath and Yoshino, 1983b), increased mitotic activity in presumptive snail hemopoietic tissues (Sullivan et al., 1984), enhanced release of lysosomal enzymes (Cheng et al., 1978; Granath and Yoshino, 1983b), increased *in vitro* phagocytosis of heterologous foreign particles (van der Knaap et al., 1987), and enhanced resistance to secondary larval challenge (Lie et al., 1975). In contrast to these stimulatory effects, trematodes also mediate suppression of phagocytic (Abdul-Salam and Michelson, 1980; van der Knaap et al., 1987) and cytotoxic (Loker et al., 1986) reactions *in vitro*, as well as interference with host responses to larval infection *in vivo* (Lie, 1982). Variation in the effects of parasites on specific hemocyte functions depends largely on the trematode–host system employed, host age and strain, infection duration, and the specific designs or conditions of experiments. Although the involvement of larval ES products has been assumed to be responsible for modulating these

hemocyte functions, our study is the first to focus at the molecular level on the interrelationship between such products and a hemocyte biochemical process with potential internal defense importance, *vis-à-vis* synthesis and release of secretory polypeptides.

Our initial observation that ES products from cultured larval schistosomes stimulated enhanced secretory protein synthesis in hemocytes of both M-line and 10-R2-OK snail strains was not unexpected, because previous studies have shown that susceptible and resistant snail hemocyte function may be similarly affected by trematode infection (e.g., Sullivan et al., 1984). The finding that 10-R2 snail hemocytes were stimulated to a greater extent than M-line cells is consistent with the hypothesis that a quantitatively higher state of cellular activation in resistant hemocytes may confer a greater cytotoxic capability on these cells when compared to hemocytes of susceptible snails. Indeed, the overall level of polypeptide synthesis by parasite-stimulated M-line hemocytes was approximately equal to that of nonstimulated control 10-R2-OK cells (data not shown).

Although 10-R2-OK hemocytes normally are at a metabolically elevated state relative to M-line cells *in vitro*, these resistant snail cells clearly are capable of being further stimulated by larval ES molecules, implying that this additional “boost” in cellular activity may be responsible for their parasite-killing efficiency. It is possible that the role of resistant snail plasma in transforming normally benign M-line hemocytes into sporocyst killer cells (Bayne et al., 1980b) is to serve as a “priming” stimulus in the sequence of events leading to full activation. This would be analogous to the sequence of tumoricidal activation found in mammalian macrophages that proceeds through several discrete stages: from responsive cell to primed cell to activated cell (Adams et al., 1985). Resistant snail hemocytes, in this analogy, would have to represent a naturally “primed” population, requiring only an activating signal(s) from encapsulated larvae to become fully cytotoxic.

An analysis of individual secretory components reveals that the protein synthetic response of hemocytes to larval ES stimulation is not a generalized one. Most striking was a nearly 3-fold increase in 1 66-kDa polypeptide, and in 10-R2-OK hemocytes, the synthesis of apparently new proteins of 156 and 105 kDa. A consistent decrease in production of at least 1 poly-

peptide upon larval ES exposure (63-kDa component in 10-R2 hemocytes) further suggests the possibility of parasite-induced "regulation" in the expression of selected effector cell polypeptides. Whether this selective synthesis of certain polypeptides is a specific tissue response of hemocytes alone has yet to be established. However, the fact that a similar response could not be elicited in a nonhemocytic Bge cell line indicates that the stimulating molecules elaborated by cultured larvae are not acting in a completely nonspecific fashion.

Finally, a preliminary assessment of the schistosome ES products responsible for modulating protein synthesis in 10-R2-OK hemocytes reveals the possible involvement of multiple larval components in stimulating synthesis of different hemocyte secretory protein groups. This conclusion is based on our observations that (1) heat treatment of parasite culture supernatants appears to abrogate enhanced synthesis of some polypeptides (e.g., Mr 180, 92, and 50 kDa), whereas the synthesis of others (e.g., Mr 220, 66, and 46 kDa) remains unaltered, and (2) that ES products of >30 kDa and those in a <10-kDa fraction stimulate synthesis of different hemocyte polypeptide groups. It appears that early schistosome larvae are capable of releasing *in vitro* an array of molecules that may be received as multiple signals by host hemocytes. The consequent secretory protein synthetic response of these host cells (which may include production of cytotoxic molecules or hemocyte regulatory factors, termed "hemokines") could well determine the degree of resistance or susceptibility exhibited in a given snail strain. Current efforts are being focussed on the isolation of specific larval ES components and determining the identity and function of the hemocyte secretory protein(s) elicited by these products.

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## THE IDENTIFICATION OF MEMBRANE GLYCOCONJUGATES IN *LEISHMANIA* SPECIES

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**ABSTRACT:** The membrane glycoconjugates of 8 different species of *Leishmania* were compared by lectin blotting. Five different lectins with various sugar specificities were examined: concanavalin A, *Lens culinaris*, *Ricinus communis*, soybean agglutinin, and peanut agglutinin. Concanavalin A and *Lens culinaris* reacted with every *Leishmania* tested. The patterns observed for these 2 lectins, as well as the various species of parasites, were different. However, a common 41,000–52,000 and a 160,000–185,000 M<sub>r</sub> component was present in almost all the parasite isolates examined. *Ricinus communis* only recognized a nondiscrete galactose-containing glycoconjugate similar to *Leishmania*-excreted factor. Soybean and peanut agglutinins reacted with a few low molecular weight parasite components. Soybean agglutinin reacted with all the *Leishmania* species tested, whereas peanut lectin only recognized 3 isolates. The latter lectin bound to discrete components migrating with the dye front and with M<sub>r</sub>'s of 35,000 and 52,000. Increased glycosylation was noted on avirulent *L. major* promastigotes and was associated with the appearance of several new peanut agglutinin-binding glycoproteins.

The protozoan parasites of the genus *Leishmania* are responsible for a wide spectrum of diseases in humans (Adler, 1964; Zuckerman and Lainson, 1977; Lainson and Shaw, 1979; Marsden, 1979). The external membrane of the parasite is at the interface with host interactions and consequently must be adapted for survival within both the hydrolytic environment of the insect gut and the phagolysosomal vacuole of the macrophage. In particular, surface glycoconjugates are of interest as carbohydrate has been demonstrated to increase protein resistance to proteolysis in various systems (Sharon, 1984) and to be involved in a range of cell–cell interactions (Hyafil et al., 1980; Berger and Armant, 1982; Pierce and Ballou, 1982). Variation in the surface glycoconjugates of *Leishmania* have been studied primarily by means of agglutination (Dwyer, 1974, 1977; Petavy et al., 1978; Doran and Herman, 1981; Hernandez, 1982; Schottelius, 1982; Schottelius and Goncalves da Costa, 1982; Ebrahimzadeh and Jones, 1983; Gueugnot et al., 1984; Wilson and Pearson, 1984; Ayesta et al., 1985). Both intra- and interspecies, as well as stage-specific variation has been demonstrated in these studies, suggesting variation in the surface glycosylation of these organisms. Variation in gly-

cosylation has also been reported to occur between avirulent and virulent organisms of *Leishmania braziliensis* (Hernandez, 1982; Ayesta et al., 1985) and between noninfective log and infective late log phase *L. major* (Sacks et al., 1985). Treatment of *L. donovani* with tunicamycin, an inhibitor of N-linked oligosaccharide synthesis rendered these parasites avirulent, suggesting a role for glycoconjugates in parasitic virulence (Nolan and Farrell, 1985). Excreted factors from *Leishmania* that have been demonstrated to be associated with the external membrane of the parasite (Jacobson et al., 1982; Kaneshiro et al., 1982) have been shown to consist primarily of carbohydrate (Slutzky et al., 1979). Evidence also suggests a regulatory role for these glycoconjugates in the uptake and survival of the parasite (Handman and Greenblatt, 1977) as well as in the modulation of the human immunological response (Londner et al., 1983). Finally, carbohydrate receptors (or lectin-like molecules) have been implicated in the uptake of the parasite by macrophages, both on the part of the macrophage (the fucose–mannose receptor) and the promastigote (Bray, 1983; Blackwell et al., 1985; Handman and Goding, 1985; Chang and Chang, 1986). However, no study identifying and comparing the surface glycoconjugates of different *Leishmania* species has been carried out to date. Such variations could be related to both the tropism and the virulence of the parasite. Western blots employing 6 different lectins, various species of *Leishmania*, and virulent/aviru-

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lent isolates were carried out. The results and analysis from these studies are presented.

# MATERIALS AND METHODS

*Leishmania* promastigotes were cultured axenically in Schneider's *Drosophila* medium (GIBCO, Grand Island, New York) supplemented with 20% fetal calf serum (GIBCO lot 26D0118). Identical stocks were used for both the lectin agglutination studies and the preparation of purified parasite membranes. *Leishmania braziliensis panamensis*, WR120 (MHOM/PA/74/WR120), and *L. major*, WR309 (MHOM/IL/79/LRC-L251), WR260B (MHOM/IL/67/Jericho II), were obtained from Dr. L. Hendricks, Walter Reed Army Institute of Research, Washington, D.C.; *L. tropica* (MHOM/IQ/LRC-L36) was kindly provided by Dr. L. Schnur, Hadassah Medical School, Jerusalem, Israel; *L. donovani infantum*, LV9 (MHOM/ET/67/L82), was obtained from Dr. M. Hommel, Liverpool School of Tropical Medicine, England; *L. braziliensis braziliensis* (MHOM/BR/75/M2903) and *L. mexicana mexicana*, L11 (MNYC/BZ/62/M379), were provided by Dr. J. Shaw, Instituto Evandro Chagas, Belem, Brazil; *L. mexicana amazonensis* (MHOM/BR/LTB0016) was obtained from Dr. P. Marsden, Universidade de Brasilia, Brazil and *L. enriettii* (MCAV/BR/Trudeau) was obtained from Dr. L. W. Poulter, Trudeau Institute, New York. *Leishmania major*, strain WR309, was derived from needle aspirates of infected animals and maintained for no more than 10 passages *in vitro*. *Leishmania major*, strain WR309-13 mo (WR309 13m), was continuously cultured and passaged twice a week for 13 mo *in vitro*. Inoculation ( $2 \times 10^7$ /site) with late log phase promastigotes of this stock into Balb/c mice resulted in lesions (2/5 mice) 2 mo later than the parent stock. In mice (3/5) with no apparent lesions, culturing of tissues excised from the site of inoculation 4 mo after injection demonstrated the presence of parasites. Cultures of spleen and liver tissue from the latter animals were negative for parasites.

Purified promastigote membranes were obtained by modifying a procedure described by Dwyer (1980). Promastigotes ( $5-10 \times 10^9$  organisms), grown as described above, were centrifuged (30 min at 1,000 g) and the pellet washed once in phosphate-buffered saline (PBS), pH 7.4 (15 min at 1,000 g). The parasites were resuspended in lysis buffer (30 ml of 0.04 M sodium chloride, 0.01 M sodium ethylenediaminetetraacetate, 0.001 M phenylmethylsulfonylfluoride, 0.001 M iodoacetamide, 0.01 M tris[hydroxymethyl]aminoethane, pH 8.0) and lysed by nitrogen cavitation (10 min, 1,500 p.s.i. at 0°C). Aliquots from all subsequent fractions were saved for enzyme analysis. The homogenate (fraction I) was centrifuged (Sorvall SS-34 rotor) for 10 min at 6,000 rpm at 4°C, and the pellet (fraction II) saved. The supernatant obtained at this step was then recentrifuged (30 min at 19,000 rpm, 4°C) and further separated into a high-speed pellet (fraction III) and supernatant. Fraction III containing the crude membranes was enriched further by ultracentrifugation on discontinuous sucrose step gradients. The crude membranes were resuspended in 0.02 M Tris-HCl, 0.003 M  $MgCl_2$ , pH 8.0 (TM) buffer containing 0.146

M sucrose, and the aggregates disrupted by 4 passages through a 22-gauge hypodermic needle. Fraction III (0.5 ml) was layered on top of a discontinuous gradient comprised of sequential layers of 1.11 M (4.0 ml), 1.52 M (4.0 ml), and 1.75 M (1.5 ml) sucrose in TM buffer. The gradients were centrifuged in an SW40 rotor at 40,000 g overnight at 4°C.

The resulting fractions banding at the interfaces between the different sucrose layers (top—fraction IV, 1.11 M/1.52 M—fraction V, and 1.52 M/1.75 M—fraction VI) were removed by syringe aspiration and frozen at  $-70^\circ C$  until further study. Prior to enzymatic analysis or use in the lectin-blotting experiments, fractions IV–VI were thawed, diluted to approximately 10% (v/v) sucrose in TM buffer, and centrifuged at 48,000 g for 30 min in a fixed angle rotor at 4°C. The pelleted membranes were washed an additional time in PBS as described and then used.

The protein concentration of each fraction was determined by the Bradford protein assay (1976). Membrane purification of each fraction relative to the initial parasite homogenate was determined by monitoring the enrichment for 2 membrane-bound enzymes, 3'-nucleotidase and 5'-nucleotidase. 5'-Nucleotidase activity was assayed in 1.0-ml reaction mixtures containing 50 mM Tris-maleate pH 7.0, 100 mM KCl, 10 mM  $MgCl_2$ , 5 mM 5'-AMP, and 10–50  $\mu g$  of protein from each fraction. The assay mixture was incubated for 30 min at 42°C and the phosphorus released measured by the Fiske-Subbarow method (Leloir and Cardini, 1957). 3'-Nucleotidase activity was determined as described by Gottlieb and Dwyer (1981). These 2 enzymes, 3'-nucleotidase and 5'-nucleotidase, are localized on the external plasma membrane in all species of *Leishmania* (Hassan and Coombs, 1987) and are markers for *Leishmania* membrane purification (Gottlieb and Dwyer, 1981). All of the fractions (IV or V) used in these studies were enriched at least 10-fold for the 3'-nucleotidase enzyme. This enzyme was a sensitive marker for *Leishmania* membrane purification and paralleled the 5'-nucleotidase activity (enriched at least 6-fold). The membrane purification procedure resulted in a loss of 5'-nucleotidase enzyme activity but did not appear to affect 3'-nucleotidase activity.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970) on 5–12% gradient gels. The Coomassie blue R-250 patterns observed for each of the fractions analyzed by lectins were essentially identical.

The lectins used in this study, concanavalin A (Con A), *Lens culinaris* agglutinin (LCA), soybean agglutinin (SBA), peanut agglutinin (PNA), wheat germ agglutinin (WGA), and *Ricinus communis*-120 (RCA), were obtained from Sigma Chemical Co. (St. Louis, Missouri). Rabbit anti-lectin IgG was purchased from E. Y. Laboratories (San Mateo, California), aliquoted, and stored at  $-20^\circ C$  until use. [ $^{125}I$ ] Con A was prepared by chloramine T oxidation and purified on Sepharose G-75 as described by Chang and Cuatrecasas (1976).

Western blots were performed using the purified *Leishmania* membrane preparations (30  $\mu g$  protein/lane) resolved on SDS-PAGE and electrophoretically transferred to nitrocellulose paper (Schleicher and Schuell, Keene, New Hampshire) according to the procedure described by Towbin et al. (1979).



TABLE I. Agglutination of *Leishmania promastigotes* by lectins.

Species	Stock	Lectin (minimum concentration $\mu\text{g/ml}$ )				
		Con A	LCA	RCA	SBA	PNA
<i>L. major</i>	WR309	2	10	0.1	1	1
	WR260B	1	0.1	0.1	10	0.1
<i>L. tropica</i>	LCR-L36	1	100	1	10	—
<i>L. donovani infantum</i>	LV9	15	100	1	7	1
<i>L. mexicana amazonensis</i>	LTB0016	10	100	500	500	—
<i>L. mexicana mexicana</i>	L11	100	500	10	—	—
<i>L. braziliensis panamensis</i>	WR120	4	500	1	500	—
<i>L. braziliensis braziliensis</i>	M2903	1	100	1	500	500
<i>L. enriettii</i>	Trudeau	0.1	—	10	100	10

The nitrocellulose paper was quenched for 1 hr at room temperature with PBS containing 2% periodic acid-treated bovine serum albumin (P-BSA) (Glass et al., 1981), 0.3% Tween 20 (Fisher Scientific, Fairlawn, New Jersey), and then washed twice with 60 ml of 0.1% Tween 20 in PBS (PBS-Tween). Con A blots were carried out using [ $^{125}\text{I}$ ] Con A ( $2 \times 10^5$  cpm/ml) in PBS-Tween, 1% P-BSA, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ ) in the presence and absence of 0.2 M  $\alpha$ -methyl-D-mannoside or 0.2 M D-mannose. After incubation for 1 hr at 25 C the nitrocellulose paper was washed extensively using PBS-Tween ( $4 \times 60$  ml) with and without sugar inhibitor, respectively, and air dried. Western blots for the additional lectins were carried out using an indirect sandwich technique (Glass et al., 1981). After electrophoretic transfer of the membranes, the nitrocellulose paper is quenched and washed as described above for [ $^{125}\text{I}$ ] Con A. The paper is then incubated for 1 hr with nonradioactive lectin (7.5  $\mu\text{g/ml}$  in PBS/Tween, 1% P-BSA) in the presence or absence of 0.2 M sugar inhibitor. The following sugars were used to inhibit lectin binding in the control strips: for SBA, N-acetyl-D-galactosamine (GalNAc) or D-galactose; for PNA, D-lactose; for LCA, D-mannose; for WGA, N-acetyl-D-glucosamine (GlcNAc), and for RCA, D-lactose or D-galactose. All of the following incubation or wash solutions for the control strips contain 0.2 M inhibiting sugar. Excess lectin was removed by washing with PBS/Tween ( $4 \times 60$  ml). The nitrocellulose paper was then reacted for 1 hr at room temperature with the appropriate rabbit anti-lectin immunoglobulin (6  $\mu\text{g/ml}$  IgG in PBS/Tween, 1% P-BSA) and washed with PBS-Tween ( $4 \times 60$  ml). The lectin-antilectin antibody sandwich bound to the paper was finally probed using donkey anti-rabbit Ig, [ $^{125}\text{I}$ ]-labeled F(ab')<sub>2</sub> fragment (Amersham Radiochemical Co., Evanston, Illinois) at  $2 \times 10^5$  cpm/ml. After 1 hr at room temperature the lectin blots were washed extensively with PBS-Tween ( $5 \times 75$  ml) and air dried. All strips were set up and exposed for autoradiography using Kodak X-omat AR film (XAR-5); Lightning-Plus intensifier screens (Dupont Neumours and Co., Wilmington, Delaware) were occasionally used.

The lectin solutions (1 mg/ml) were prepared in PBS with and without 0.1 M sugar inhibitor. Standard dilutions of the lectin solutions were made and pipetted (50  $\mu\text{l}$ ) into 96-well V-bottom microtiter plates (Flow Laboratories, Inc.). The final concentrations used in

the agglutination studies after addition of the promastigotes were 500, 100, 10, 1, and 0.1  $\mu\text{g}$  lectin/ml.

Promastigotes were centrifuged (10 min  $\times$  1,000 g at 4 C) and the pellet was resuspended in PBS (15 ml) and washed by centrifugation ( $2 \times 15$  ml). The number of organisms was counted and adjusted to  $2 \times 10^8/\text{ml}$  in PBS. Live promastigotes ( $5 \times 10^6$  organism) were added to each well. After an overnight incubation at 4 C the plates were scored for agglutination both microscopically and macroscopically. Scoring for microscopic agglutination was carried out essentially as described by Dwyer (1974).

## RESULTS

### Agglutination

Nine different stocks of *Leishmania* belonging to 8 different species and subspecies of the parasite were examined. The minimum concentration of lectin found to cause agglutination of the promastigotes is listed in Table I. All of the *Leishmania* are strongly agglutinated by Con A, which belongs to the D-mannose/D-glucose-binding group of lectins. LCA, which also belongs to this lectin group, shows a pattern very similar to Con A, with the exception of *L. enriettii*, which is not agglutinated by LCA. In addition, higher concentrations of LCA than Con A (except for WR260B) were required to cause promastigote agglutination. Among the lectins belonging to the D-galactose-binding family, only RCA strongly agglutinates all of the *Leishmania* species examined. The remaining 2 lectins, SBA and PNA, which also belong to this group, show considerable variability in agglutination between the different species of *Leishmania*. Although the 2 stocks of *L. major* (WR309 and WR260B) show agglutination at low titers with both PNA and SBA, only *L. enriettii* among the remaining species tested was also agglutinated by both these lectins, at  $<500$   $\mu\text{g/ml}$ . The 2 *L. braziliensis* sp.



stocks (WR120 and M2903) examined are poorly agglutinated by these lectins (500  $\mu\text{g/ml}$ ), and of the 2 *L. mexicana* sp. only LTB0016 is marginally agglutinated by SBA (500  $\mu\text{g/ml}$ ). L11 is not agglutinated by either SBA or PNA. *Leishmania tropica* is only agglutinated by SBA (10  $\mu\text{g/ml}$ ) and *L. donovani infantum* is only agglutinated by PNA (1  $\mu\text{g/ml}$ ). None of the species, except for *L. enriettii* was agglutinated by WGA, a GlcNAc-binding lectin. This species shows a marginal reaction with WGA at high concentrations, (500  $\mu\text{g/ml}$ ).

### Lectin blotting

Con A and LCA have similar sugar specificities and recognize similar glycoproteins (Goldstein and Hayes, 1978; Lis and Sharon, 1984). The binding of LCA and Con A to purified membranes of several *Leishmania* species and subspecies is shown in Figures 1 and 2. The same stocks used for the agglutination studies, described above, were used in the lectin blots. All of the *Leishmania* stocks examined contain glycoproteins that bind LCA. The binding was specifically inhibited by D-mannose (Fig. 2B). This lectin recognizes multiple components in almost every stock with molecular weights ( $M_r$ ) ranging from 27,000 to 200,000. Two exceptions were found, *L. b. braziliensis* (M2903) and *L. enriettii*, where the lectin only reacts with a single component,  $M_r = 52,000$  and 200,000, respectively. Comparisons between the LCA-binding membrane glycoproteins for the various *Leishmania* examined by this technique show, in general, that little similarity exists among the different species and subspecies. The pattern of lectin reactivity with *L. b. panamensis* (lane 3) bears no resemblance to *L. b. braziliensis* (lane 9). In the case of *L. tropica* (lane 2) the lectins bind to several glycoproteins absent from both *L. major* stocks (lanes 1 and 5) examined. Only in the case of *L. m. amazonensis*, lane 6, and *L. m. mexicana*, lane 8, are the overall patterns obtained essentially identical.

Major LCA-binding glycoproteins,  $M_r$  41,000–52,000, are present in *L. b. braziliensis* (48,000 and 50,000), *L. m. amazonensis* (41,000 and 48,000), and *L. tropica* (44,000). Weakly staining bands in this molecular weight range are also found in *L. donovani infantum* (48,000), *L. major* (48,000), and *L. b. panamensis* (48,000 and 52,000). Similarly, a high molecular weight doublet or triplet (160,000, 175,000, and 185,000) seems to be present in 7 out of 9 of the stocks

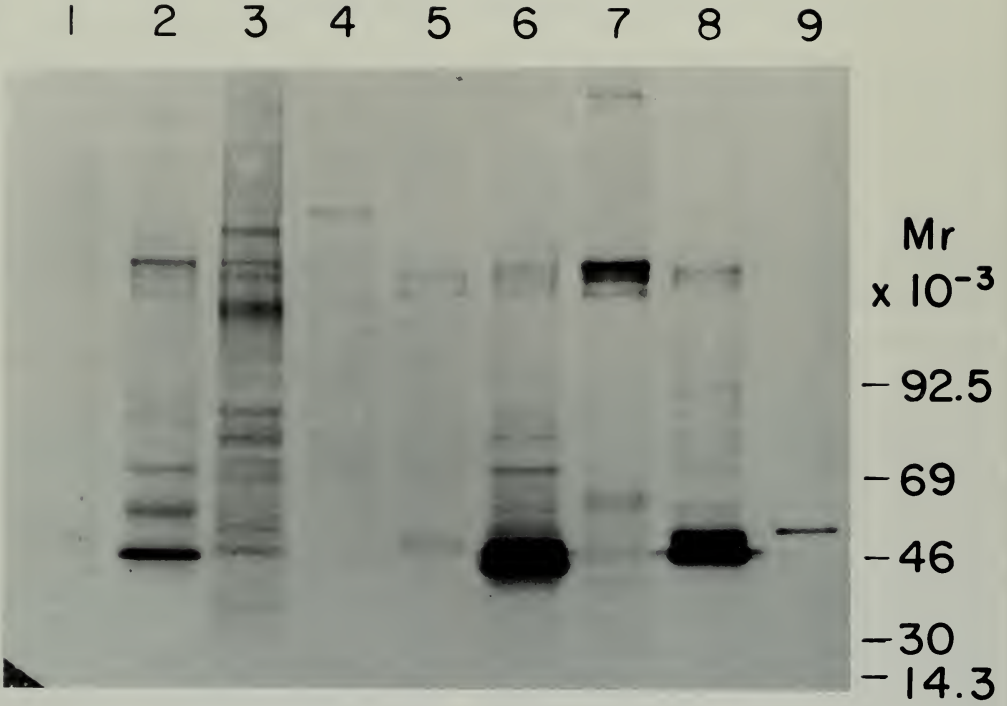
examined, the exceptions being *L. enriettii* and *L. b. braziliensis*.

Of all the lectins studied, only in the case of Con A did blots using the triple sandwich technique have extremely high levels of apparently nonspecific, nonsugar-inhibitable binding to the nitrocellulose. Therefore, direct lectin blots with [ $^{125}\text{I}$ ] Con A (Fig. 2) on identical *Leishmania* membrane preparations were carried out. Blots using the direct staining procedure resulted in highly specific, sugar-inhibitable Con A binding to glycoproteins identified by the triple sandwich technique. The major bands identified by the direct lectin blot were also visible with the sandwich technique. However, many of the weaker bands observed using the latter technique were not apparent in autoradiograms obtained from a direct blot. The direct lectin blot appears to be less sensitive, although apparently more specific, than lectin blots for Con A using the triple sandwich technique.

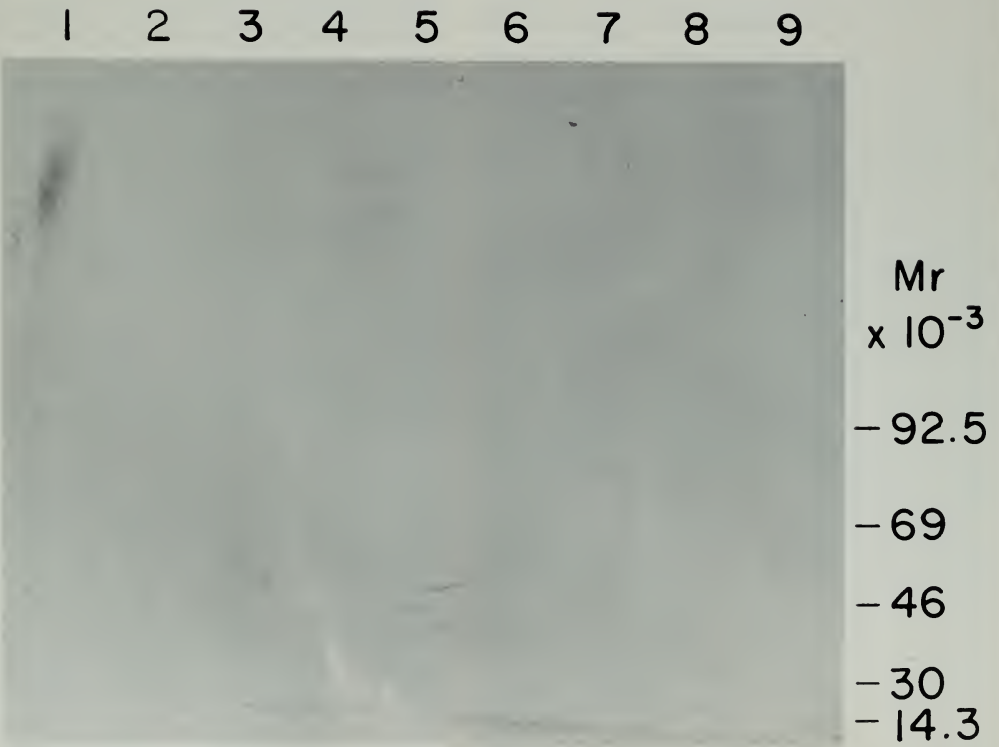
As might be expected, the major glycoproteins recognized by Con A are similar to those obtained for LCA. Like LCA, Con A also reacts with a 41,000–52,000 family of glycoproteins (7 out of 9 stocks examined) and high  $M_r$  doublet triplet (160,000, 175,000, and 185,000). However, the high molecular weight components are only seen in the direct blot with 2 isolates (lanes 5 and 6) when Con A is used. Several additional glycoproteins absent in the LCA blots are recognized by Con A. With *L. d. infantum* (lane 7) 2 new bands (34,000 and 39,000) are apparent. These glycoproteins, in addition to the 48,000, 57,000, and 61,000 bands that are recognized by LCA, appear to be the major Con A-binding components for this species. Con A also recognized 2 additional glycoproteins 76,000 and 92,000, in *L. major* (lane 1), and using the triple sandwich technique (data not shown) several lectin-specific high molecular weight components in *L. b. braziliensis*.

The lectins RCA, SBA, and PNA all belong to the D-galactose-binding family of lectins. However, these lectins exhibit different sugar specificities for complex sugars (Goldstein and Hayes, 1978; Lis and Sharon, 1984; Molan and Farrell, 1985; McMahon-Pratt and Jaffe, 1986) and react with different *Leishmania* membrane components on lectin blots. The triple sandwich procedure was used for RCA, SBA, and PNA. RCA recognizes glycoconjugate components on 7 of 9 leishmanial parasites examined (Fig. 3A). Following long exposure of the strips, *L. tropica*

A.



B.



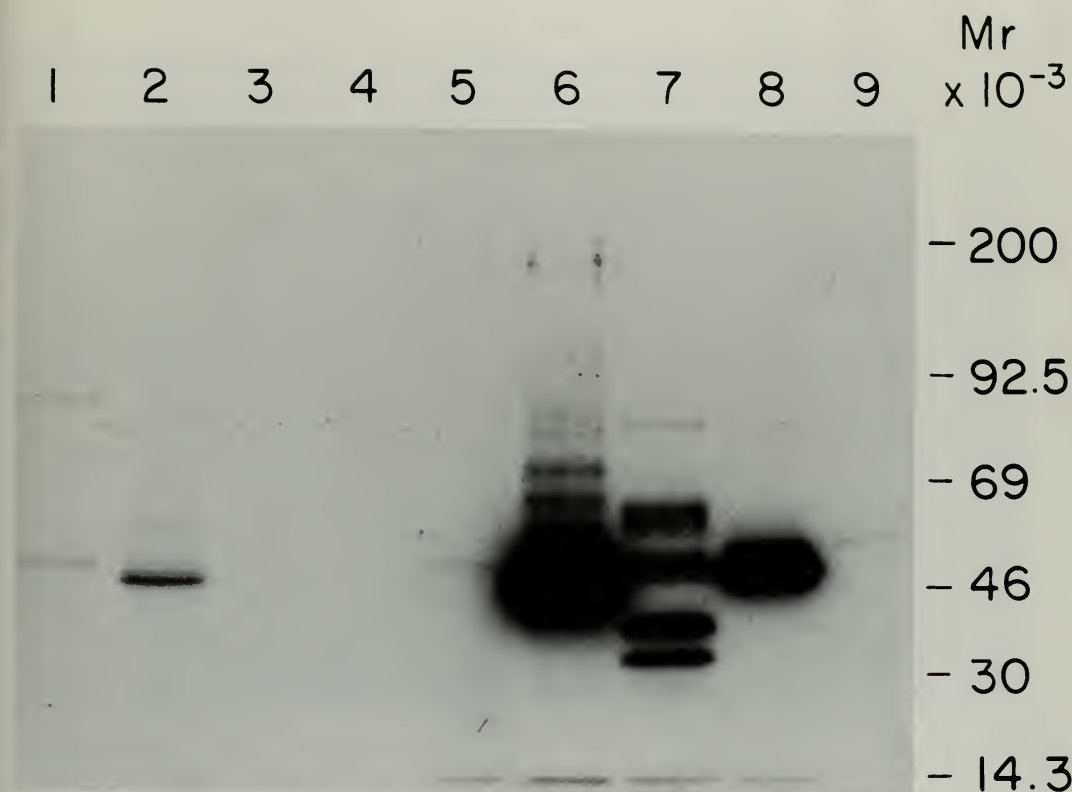


FIGURE 2. Western blot analysis of *Leishmania* promastigote membrane preparations probed with [ $^{125}$ I] concanavalin A. The *Leishmania* strains in each lane are the same as for Figure 1.

(LRC-L36) was the only stock where no reaction was observed. With 6 of the 8 positive stocks, binding to diffuse bands of either low molecular weight 14,300–60,000 and/or high molecular weight 150,000–240,000 was present. The diffuse staining seen with RCA blots is a property of the glycoconjugates and not a gel artifact, because identical western blots show discrete binding patterns when reprobed with LCA. Variations between species in the exact upper and lower  $M_r$  for each diffuse band were found. Two discrete bands ( $M_r$  42,000 and 47,000) appear with *L. m. amazonensis* following long exposure of the autoradiogram (data now shown). The binding of RCA to these glycoconjugates was inhibited specifically by 0.2 M D-galactose.

Long exposures of the RCA sugar control blot (no diffuse bands are present) revealed several discrete 0.2 M D-galactose noninhibitable components (Fig. 3B) that were masked by the more intense RCA-binding smears in the absence of sugar. Surprisingly, it was found that these bands were very similar to *Leishmania* glycoprotein components identified by SBA (Fig. 4, lanes 5–8). The binding of SBA to these components could be specifically blocked if 0.01 M GalNAc, a sugar inhibitor for SBA, was included. SBA reacts weakly with the stocks studied and the bands are only apparent following very long exposures of the autoradiogram. This is responsible for the high backgrounds observed. Each stock contains a different set of the SBA-binding glycoproteins,

FIGURE 1. *Leishmania* promastigote membrane preparations probed with *Lens culinaris* lectin using the triple sandwich technique in the absence (A) or presence (B) of 0.2 M  $\alpha$ -methyl-D-mannoside. The *Leishmania* strains in each lane are as follows: (1) WR309, *L. major*, (2) LRC-L36, *L. tropica*, (3) WR120, *L. braziliensis panamensis*, (4) *L. enriettii*, (5) WR260B, *L. major*, (6) LTB0016, *L. mexicana amazonensis*, (7) LV9, *L. donovani infantum*, (8) L11, *L. mexicana mexicana*, and (9) M2903, *L. braziliensis braziliensis*.



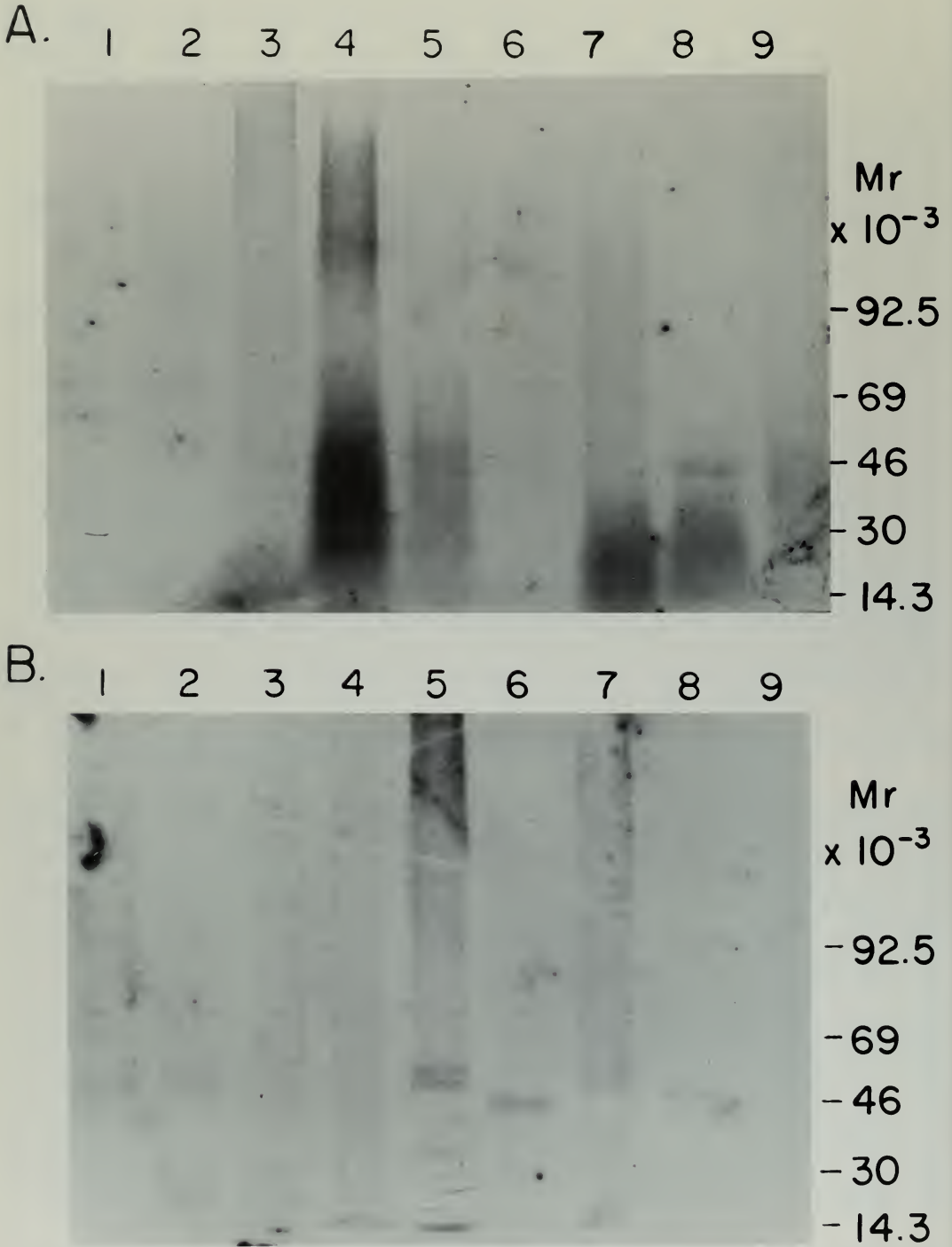


FIGURE 3. Western blot analysis of *Leishmania* promastigote membrane preparations probed with *Ricinus communis* 120 agglutinin using the triple sandwich technique in the absence (A) or presence (B) of 0.2 M D-galactose. The *Leishmania* strains in each lane are the same as for Figure 1.

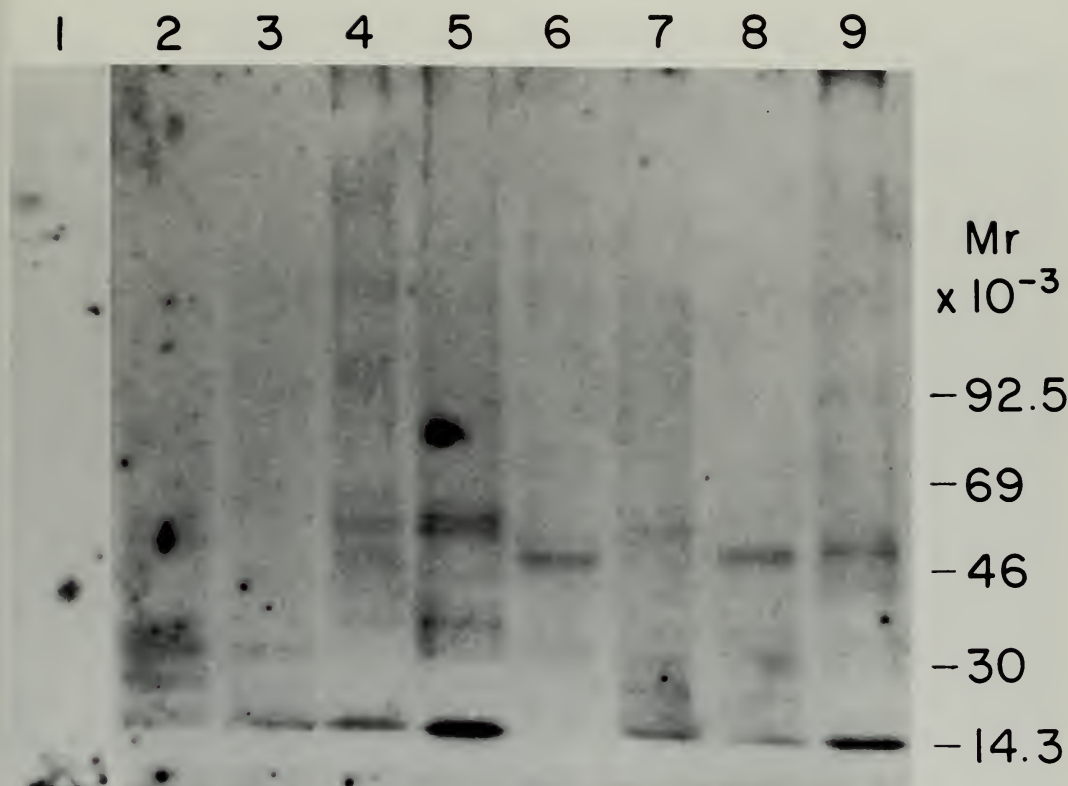


FIGURE 4. Western blot analysis of *Leishmania* membrane preparations probed with soybean agglutinin using the triple sandwich technique. The *Leishmania* strains in each lane are the same as for Figure 1.

from 1 out of 6 glycoproteins (*L. m. amazonensis*, *L. m. mexicana*, *L. major* WR309, *L. b. panamensis*, and *L. b. braziliensis*), to 3 or 4 of the 6 glycoproteins observed (*L. tropica*, *L. major* WR260B, *L. d. infantum*, and *L. enriettii*). SBA also binds to molecules migrating with the dye front in 6 out of 9 *Leishmania* stocks.

PNA, which shows the most highly restricted specificity of all the D-galactose lectins tested (Gal $\beta$ 4GlcNAc and Gal $\beta$ 3GlcNAc), only binds to glycoproteins from 3 of the 9 stocks studied (Fig. 5). This lectin reacts with glycoconjugates from *L. b. panamensis*, *L. enriettii*, and *L. major* (WR260B), and the binding is specifically inhibited by 0.2 M lactose. Two of the 3 species have a strong PNA-binding membrane component that migrates with the dye front. This glycoconjugate is the major PNA-binding component for *L. enriettii* and *L. b. panamensis*, although additional bands are visible for *L. enriettii* and *L. b. panamensis* on long exposure. The rapidly migrating glycolipid is a very minor component in *L. major*, visible only on overexposure of the blot (not

shown). The major PNA-binding glycoconjugates in this stock are 3 bands of 35,000, 49,000, and 52,000.

Lectin-binding components of *L. major* WR309 promastigotes that had been continuously cultured for 13 mo (Fig. 6) were compared to promastigotes derived recently from infected mice (Figs. 1, 2, 4, 5). Binding to glycoconjugates recognized by LCA and Con A was much more pronounced in WR309 13m. In addition, PNA that did not react with any glycoconjugates of the pathogenic WR309 promastigotes detected several distinct components in the nonpathogenic WR309 13m with  $M_r$  28,000, 38,000, and a broad diffuse band ( $M_r$  40,000–80,000).

## DISCUSSION

Glycoconjugates have been used as a means of *Leishmania* classification. Antibodies raised to "excreted factor," which has been characterized (Schnur, 1982) as a glycoconjugate, are able to distinguish among the Old World species on the basis of an Ouchterlony reaction. These se-

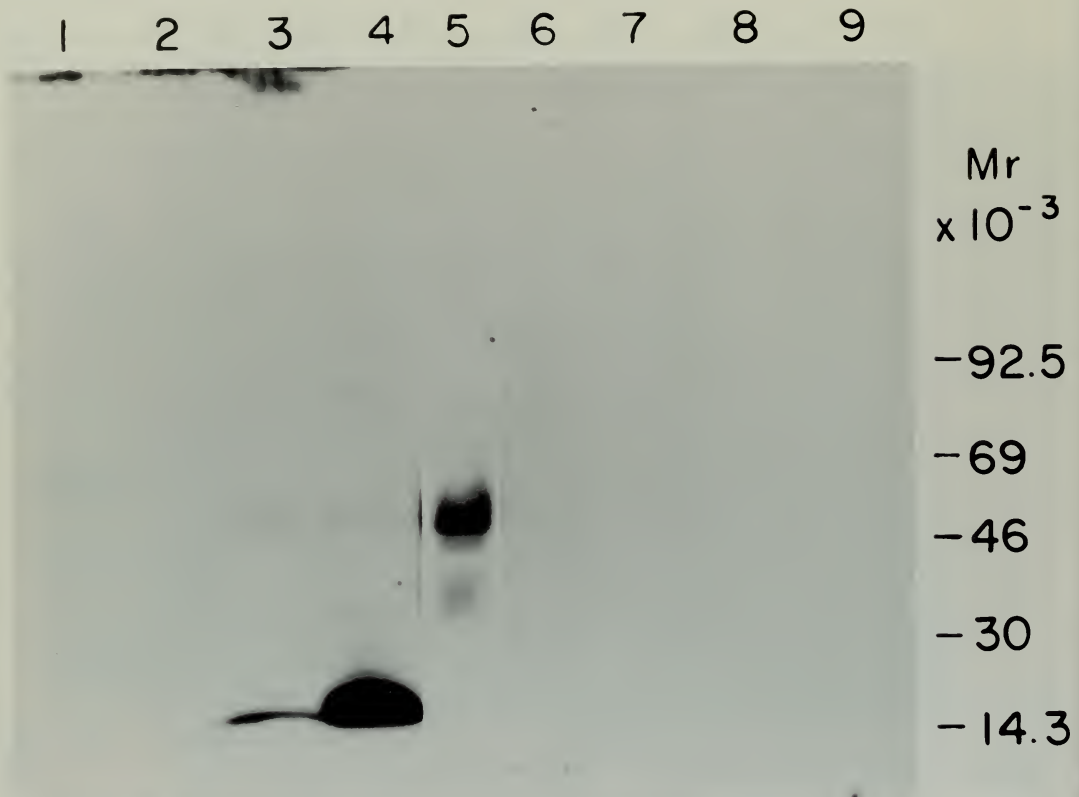


FIGURE 5. Western blot analysis of *Leishmania* membrane preparations probed with peanut agglutinin using the triple sandwich technique. The *Leishmania* strains in each lane are the same as for Figure 1.

rotypes have been correlated with lectin-mediated agglutination (Hernandez, 1982). Promastigotes of the  $A_1$  (*L. tropica*) and  $A_2$  (*L. major*) subserotypes can be distinguished by means of Con A-induced agglutination, whereas RCA-induced agglutination can be used to discern differences between the  $A_1$  and  $A_2$  subserotypes and also between the  $B_1$  and  $B_2$  subserotypes. SBA differentiates between the A and B serotypes.

The agglutination of *Leishmania* by carbohydrate-binding lectins has been extensively examined. Previous lectin studies of *Leishmania* species (Dwyer, 1974; Doran and Herman, 1981; Hernandez, 1982; Schottelius, 1982; Schottelius and Goncalves da Costa, 1982; Gueugnot et al., 1984) have found in general that promastigotes of *L. braziliensis*, *L. donovani*, *L. major*, *L. tropica*, *L. mexicana*, and *L. aethiopica* are agglutinated by Con A and *Ricinus communis*. Soybean (*Glycine max*) lectin agglutinates most stocks of *L. major* and *L. tropica* but does not in general react with stocks of *L. aethiopica*, *L. donovani*,

or *L. braziliensis*. Peanut (*Arachis hypogaea*) lectin does not agglutinate *L. braziliensis* or *L. mexicana* but does agglutinate most stocks of *L. donovani*. Variable reactions were observed with the peanut lectin and members of the *tropica* complex. The agglutination results presented in this paper are consistent with previously reported studies. However, there does not seem to be a strong correlation between the ability of a particular species of *Leishmania* to be agglutinated and the Western blot pattern observed for the same species and lectin. This type of phenomenon has been found in studies using erythrocytes where glycolipids are involved (Lis et al., 1982).

Lectin blot analyses presented in this study reveal that the membrane components recognized by the lectins Con A and LCA are variable among the species of *Leishmania*. However a major component migrating with an  $M_r$  of approximately 50,000 was observed in most stocks studied (7/9) and probably corresponds to the major  $^{125}\text{I}$ -surface-labeled glycoprotein of *Leish-*



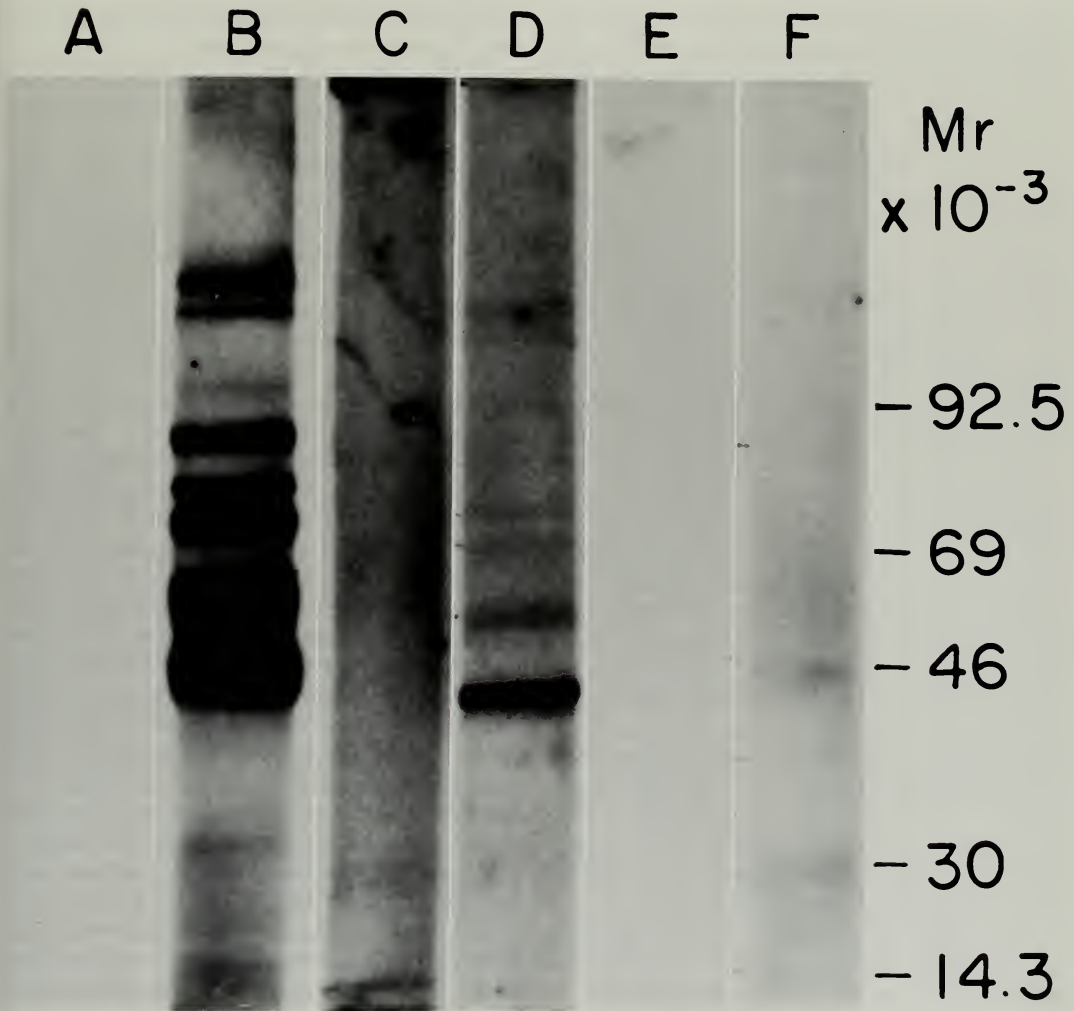


FIGURE 6. Western blot analysis of strain WR309 13m (*L. major*), continuously cultured *in vitro* for 13 mo and probed with concanavalin A (lanes A and B), *Lens culinaris* lectin (lanes C and D), and peanut agglutinin (lanes E and F). Blots were carried out either in the presence (lanes A, C, and E) or the absence (lanes B, D, and F) of 0.2 M of the appropriate sugar.

*mania* (Alexander and Russell, 1985), which appears to be ubiquitous in New and Old World *Leishmania*. By SDS-PAGE analysis this glycoprotein has a molecular weight of 63,000 or 50,000 under reducing or nonreducing conditions, respectively (Bouvier et al., 1985; McMahon and Jaffe, 1986). The purified component from *L. m. amazonensis* contains mannose, GlcNAc, and GalNAc (Alexander and Russell, 1985). A similar glycoprotein from *L. donovani*, *L. major*, and *L. m. amazonensis* binds [<sup>125</sup>I] Con A (Jaffe, unpubl. data) and purified material from *L. m. amazonensis* binds to LCA-sepharose 4B (Chang and Chang, 1986). Treatment of this af-

finity-purified material with endoglycosidase H indicates the presence of 3–4 oligosaccharide sidechains (Jaffe, unpubl. data; Chang and Chang, 1986).

Recently, the mannose/fucose receptor on macrophages has been shown to be involved in the binding and uptake of promastigotes by macrophages (Alexander and Russell, 1985; Blackwell et al., 1985; Chang and Chang, 1986). The major glycoprotein appears to bind to one of the ligands recognized by this macrophage receptor (Alexander and Russell, 1985; Chang and Chang, 1986). Thus, the prominent 41,000–52,000 glycoprotein family observed in all *Leishmania* with

both Con A and LCA is probably due to the important role of these glycoconjugates in parasite invasion of macrophages.

The multiple glycoconjugates recognized in *L. donovani* infantum stock LV9 by Con A and LCA are similar to those observed by Dwyer using a Sudan strain with fluorescein isothiocyanate-derivatized lectin (Dwyer, 1981) and by Lepay et al. (1983) in studies employing Con A-sepharose-fractionated surface-radioiodinated *L. donovani* promastigotes. These data suggest that specific glycoconjugates may be distinctive and characteristic of species of *Leishmania*, although further work is necessary in order to confirm these observations. However, it should be noted that the lectin-binding patterns observed for the 2 *L. major* stocks studied were not identical and that heterogeneity has been reported among various strains of *L. donovani* and *L. major* examined by both lectin agglutination (Hernandez, 1982), fluorescent-derivatized lectin binding (Jacobson and Schnur, 1987), and isoenzyme analyses (Kreutzer et al., 1983). We have also noted variations in lectin binding by Western blot analysis when isolates of *L. major* were examined (Jaffe, unpubl. data). The strong reactions seen for the glycoconjugates of *L. mexicana* spp. (strains LTB0016 and L11) are consistent with the analyses of Parodi et al. (1984) demonstrating that oligosaccharide composition of N-linked glycoproteins is primarily Man<sub>6</sub>GlcNAc<sub>2</sub>.

Interestingly, LCA, which to date has not been as extensively used as Con A for *Leishmania* agglutination studies, readily distinguishes *L. major* from other species of *Leishmania*. Although Con A and LCA have similar binding specificities for the simple monosaccharides, they differ in their affinities toward more complex oligosaccharides and glycoproteins (Lis and Sharon, 1984). In the case of *Leishmania* membranes it appears that LCA and Con A bind to several similar components. However, Con A appears to react preferentially with the low molecular weight components (34,000–61,000), whereas LCA binds more to the high molecular weight components (>100,000).

Several high molecular weight components (160,000, 175,000, 185,000) appear to be relatively conserved in 6/8 species studied. Because these glycoproteins are conserved it is likely they also play an important functional role for the promastigote. Initial depletion of the low molecular weight Con A-binding glycoproteins

should allow purification of these high molecular weight parasite components for further study.

RCA recognizes D-galactose and GalNAc. We have shown that this lectin reacts with a broad band of membrane-associated component(s). This pattern is similar to that observed on SDS-PAGE by Turco and coworkers (1984) for a phosphorylated galactosyl- $\beta$ -mannose polysaccharide from *Leishmania donovani*; by Handman et al. (1984) for isolated excreted factor from *L. major*, a polysaccharide containing glucose, galactose, phosphate, and sulphate; and by Palatnik et al. (1985) for a lipopeptidophosphomannan containing mannose and galactose isolated from *L. adleri*. Excreted factor has been associated with the uptake and survival *in vitro* of *Leishmania* by and in macrophages (Handman and Greenblatt, 1977; Handman and Goding, 1985), as well as in the modulation of the human immunological response (Londner et al., 1983) and is known to be present in a membrane-bound form (Handman and Goding, 1985). Although the monoclonal antibodies used for excreted factor purification were species specific and recognized only the polysaccharide of *L. major*, the studies carried out here with RCA identify similar galactose-containing components in other species of *Leishmania*. Excreted factor is probably the primary component responsible for the pan-*Leishmania* agglutination observed with this lectin. In the case of *L. major* WR309 or *L. tropica* and *L. m. amazonensis*, respectively, only an extremely weak broad high or low molecular weight band was noted. The apparent low level of polysaccharide components in these strains may be due to a structural variation that precludes strong RCA binding or to reduced amounts of ligand in the fraction examined.

It should be noted, however, that increased RCA agglutination has been associated with non-infective log phase promastigotes of *L. major* (Sacks et al., 1985) and *L. donovani* (Doran and Herman, 1981). *Leishmania major* WR309 is highly infective causing a disseminated disease in BALB/c mice with less than  $10^4$  promastigotes. Infection of BALB/c mice with *L. major* WR260B ( $10^8$  parasites) causes nonprogressive lesions in only a fraction of the mice. Consequently, it seems possible that excreted factor may vary between the pathogenic and nonpathogenic promastigotes. Indeed, one stock of *L. major* that does not produce excreted factor has been isolated; this parasite is avirulent (Handman, pers.



comm.). Interestingly, RCA blots performed in the presence of sugar inhibitor revealed discrete bands. Patterns observed were very similar to those found with SBA. Unlike RCA, the binding for SBA was specifically inhibited by D-galactose. Consequently, these discrete components are probably recognized specifically by RCA, but are tightly associated with the lectin and not readily inhibited by the simple sugar.

Peanut lectin, which is inhibited by Gal $\beta$ 3GalNAc, only binds to 3 of the *Leishmania* examined. Recently, peanut lectin has been reported to differentially bind to the amastigote stage of *L. donovani* (Wilson and Pearson, 1984). In the case of *L. major*, noninfective log phase promastigotes but not infective late log phase promastigotes have been reported to be agglutinated by this lectin (Sacks et al., 1985). Antiserum raised to log phase promastigotes recognized, by Western blot analysis, 2 molecules ( $M_r$  28,000 and 30,000) specific for the noninfective organisms. However, these molecules were not observed in immunoprecipitation experiments employing surface-radioiodinated promastigotes (Sacks et al., 1985). Lectin blotting with PNA in our study found that this lectin selectively binds to 28,000, 37,000, and 48,000  $M_r$  membrane components only found in nonpathogenic WR309 organisms. This result suggests that the PNA-binding components may be related to those observed by Sacks et al. (1985). If so, the 2 low  $M_r$  components present in noninfective log stage organisms are membrane associated and contain carbohydrate consistent with PNA-binding sites. It is also of interest that the weakly pathogenic *L. major* strain WR260B displays PNA-binding components in this same  $M_r$  range.

Lectin-binding analyses employing Western blot techniques have resulted in the identification of a number of glycoconjugates associated with the surface membranes of *Leishmania*. Some of these components, especially those seen by Con A and LCA, are common to the various species of *Leishmania* and may represent conserved glycoproteins of particular importance to the parasites. In addition, the presence of certain glycoconjugates have been found to correlate with pathogenicity of the organism.

#### ACKNOWLEDGMENTS

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## CHARACTERIZATION OF THE N- AND O-LINKED OLIGOSACCHARIDES IN GLYCOPROTEINS SYNTHESIZED BY *SCHISTOSOMA MANSONI* SCHISTOSOMULA

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**ABSTRACT:** This report describes the structural analyses of the O- and N-linked oligosaccharides contained in glycoproteins synthesized by 48-hr-old *Schistosoma mansoni* schistosomula. Schistosomula were prepared by mechanical transformation of cercariae and were then incubated in media containing either [2-<sup>3</sup>H] mannose, [6-<sup>3</sup>H]glucosamine, or [6-<sup>3</sup>H]galactose to metabolically radiolabel the oligosaccharide moieties of newly synthesized glycoproteins. Analysis by SDS-polyacrylamide gel electrophoresis and fluorography demonstrated that many glycoproteins were metabolically radiolabeled with the radioactive mannose and glucosamine precursors, whereas few glycoproteins were labeled by the radioactive galactose precursor.

Glycopeptides were prepared from the radiolabeled glycoproteins by digestion with pronase and fractionated by chromatography on columns of concanavalin A-Sepharose and pea lectin-agarose. The structures of the oligosaccharide chains in the glycopeptides were analyzed by a variety of techniques.

The major O-linked sugars were not bound by concanavalin A-Sepharose and consisted of simple O-linked monosaccharides that were terminal O-linked N-acetylglucosamine, the minor type, and terminal O-linked N-acetylglucosamine, the major type. The N-linked oligosaccharides were found to consist of high mannose- and complex-type chains. The high mannose-type N-linked chains, which were bound with high affinity by concanavalin A-Sepharose, ranged in size from Man<sub>6</sub>GlcNAc<sub>2</sub> to Man<sub>9</sub>GlcNAc<sub>2</sub>. The complex-type chains contained mannose, fucose, N-acetylglucosamine, and N-acetylglucosamine. No sialic acid was present in any metabolically radiolabeled glycoproteins from schistosomula.

The human blood fluke *Schistosoma mansoni* synthesizes numerous glycoproteins (Norden and Strand, 1984; MacGregor et al., 1985; Hayunga and Sumner, 1986a; Nyame et al., 1987). Some studies indicate that the oligosaccharide moieties of these glycoproteins are antigenic in infected hosts (Omer-Ali et al., 1986; Weiss et al., 1986; Grzych et al., 1987). However, the roles of the glycoproteins and their sugar moieties in schistosome development and survival in its host are not well understood.

Some previous studies indicate that schistosomula and adult schistosomes display different affinities for various plant lectins (Simpson and Smithers, 1980; Simpson et al., 1983), suggesting that the oligosaccharide moieties of schistosome glycoproteins undergo changes during the development from schistosomula to adult schistosomes. It has also been reported that when schistosomula develop to adults, some glycoproteins present in schistosomula disappear and new species of glycoproteins are synthesized in adults (Payares and Simpson, 1985; Hayunga and Sumner, 1986c). These studies suggest that the oli-

gosaccharide moieties of schistosome glycoproteins might be involved in the developmental changes of the organism and might also be involved in the age-dependent susceptibility to immunity displayed by *Schistosoma mansoni* (Bickle and Ford, 1982). To eventually examine the role of schistosome glycoproteins and their oligosaccharide moieties in the development and survival of the parasite in their hosts, we have begun to characterize the structures of the oligosaccharide moieties in their glycoproteins (Nyame et al., 1987, 1988). In this paper, we report on the O- and N-linked sugars in glycoproteins synthesized by 48-hr-old *S. mansoni* schistosomula.

### MATERIALS AND METHODS

#### Materials

Concanavalin A-Sepharose (Con A-Sepharose) was obtained from Pharmacia P-L Biochemicals. Pea lectin-agarose was purchased from E-Y Laboratories. Alpha-methylglucoside,  $\alpha$ -methylmannoside, Sephadex G-25-80, QAE-Sephadex, Amberlite MB-3, and sodium borohydride were purchased from Sigma Chemical Co. Dowex 50 (H<sup>+</sup> form) was obtained from Baker Chemical Co. Materials for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography were purchased from Bio-Rad Laboratories and NEN Research Products, respectively. Pronase was obtained from Calbiochem. [6-<sup>3</sup>H]glucosamine (40 Ci/mmol), [2-<sup>3</sup>H]mannose (21 Ci/mmol), and [6-<sup>3</sup>H]galactose

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(25 Ci/mmol) were purchased from Amersham. UDP-N-acetylgalactosamine [ $^{14}\text{C}$ ] galactosamine (47.2 mCi/mmol) and UDP-N-acetylglucosamine [ $^3\text{H}$ ]glucosamine (20.4 Ci/mmol) were obtained from New England Nuclear Corp. The radioactive standards N- $^{3\text{H}}$ acetylglucosamine and N- $^{14}\text{C}$ acetylgalactosamine were prepared by mild acid hydrolysis of the radioactive sugar nucleotides in 10 mM HCl at 100 C for 10 min. N- $^{3\text{H}}$ acetylglucosaminitol and N- $^{14}\text{C}$ acetylgalactosaminitol were prepared by reduction of the appropriate reducing sugars with sodium borohydride. The reduced sugars were purified by passage over a column of Amberlite mixed-bed ion-exchange resin in water. The disaccharide standard Gal1,3GalNAc (Gal, Galactose; GalNAc, N-acetylgalactosamine) was a gift from Dr. W. Gielen (University of Köln, West Germany). The reduced form of this disaccharide was prepared by reduction with  $\text{NaBH}_4$ . Endo- $\beta$ -N-acetylglucosaminidase H (Endo H) was purchased from Miles Scientific. The radioactive standards Man $_{5-9}$ -GlcNAc $_1$  (Man, Mannose; GlcNAc, N-acetylglucosamine) were prepared from the mouse lymphoma cell line BW5147, after incubating  $2 \times 10^6$  cells in media containing [ $^3\text{H}$ ]mannose, as described previously (Cummings and Kornfeld, 1982). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from GIBCO and baboon serum was purchased from the Southwest Foundation for Biomedical Research, San Antonio, Texas.

#### Preparation of schistosomula by mechanical transformation

Cercariae, KEB strain (Kenyan, baboon passaged), were obtained from infected *Biomphalaria pfeifferi* (Tanzanian strain) snails. The cercariae suspension was concentrated by spinning at 1,500 rpm for 2 min. The pellet was washed once with DMEM and resuspended in DMEM and transformed into schistosomula by pushing them through 2 syringes connected by an emulsification needle (Basch, 1981). The transformed schistosomula were washed 2 times with DMEM to remove snail debris and mucus. Released tails and other debris were removed from the schistosomula preparation by centrifugation over a 60% Percoll gradient as modified by Dr. P. T. LoVerde (pers. comm.). The pellet that contained schistosomula bodies was washed 2 times to remove Percoll. Residual glycocalyx was removed by incubation in a shaking water bath at 37 C for 90 min (Ramalho-Pinto et al., 1974).

#### Metabolic radiolabeling of mechanically transformed schistosomula with radioactive precursor sugars

Approximately 5,000 schistosomula were labeled *in vitro* by incubation with 4 mCi/ml of either [ $^3\text{H}$ ]mannose, [ $^3\text{H}$ ]glucosamine, or [ $^3\text{H}$ ]galactose in DMEM containing 5% normal baboon serum, garamycin (50 g/ml), and fungizone (0.024 g/ml) in a final volume of 0.25 ml for 48 hr. Incubations were performed at 37 C in a humidified incubator containing 5%  $\text{CO}_2$ . The schistosomula were motile and viable at the end of the incubation, and the culture was free of bacterial and fungal contamination.

#### SDS-PAGE of radiolabeled glycoproteins from schistosomula

Radiolabeled schistosomula were washed twice in DMEM and sonicated with a Branson sonifier. The radiolabeled glycoproteins were then analyzed by 1-dimensional gel electrophoresis on a polyacrylamide gradient (5–20% acrylamide) slab gel in SDS under reducing conditions (Laemmli, 1970) and visualized by fluorography (Cummings et al., 1983).

#### Preparation of radiolabeled glycoproteins from schistosomula

Radiolabeled schistosomula were washed twice in DMEM and then sonicated in lysis buffer (0.1 M Tris-HCl, pH 8.0, containing 1 mM  $\text{CaCl}_2$ ). The homogenate was extracted 3 times with 20 volumes of chloroform-methanol (2:1) to remove lipids. The residual protein was dried under a gentle stream of nitrogen and incubated with 10 mg/ml pronase in lysis buffer for 24 hr at 60 C in a toluene atmosphere. After digestion with pronase, the sample was boiled for 5 min and desalted on a column (1  $\times$  50 cm) of Sephadex G-25 in 7% 1-propanol. The radiolabeled glycopeptides, which were recovered in the void fractions, were pooled and dried in a shaker bath evaporator under vacuum.

#### Column chromatography

Radiolabeled glycopeptides were fractionated on 2-ml columns (0.7  $\times$  5 cm) of Con A-Sepharose at room temperature (Cummings and Kornfeld, 1982). Glycopeptides bound to the column were eluted first with 10 mM  $\alpha$ -methylglucoside followed by 100 mM  $\alpha$ -methylmannoside (buffer at 60 C). Fractions of 2 ml were collected from the column and aliquots were taken and mixed with ScintiVerse I (Fisher) for determining radioactivity in a liquid scintillation counter. Chromatography of glycopeptides on pea lectin-agarose was performed on a 2-ml column (0.7  $\times$  5 ml) at room temperature, and 2-ml fractions were collected. Bound glycopeptides were eluted with 10 mM  $\alpha$ -methylglucoside followed by 500 mM  $\alpha$ -methylmannoside. Ion-exchange chromatography of glycopeptides was conducted on 2-ml columns (0.7  $\times$  5 cm) of QAE-Sephadex in 2 mM Tris-base (Varki and Kornfeld, 1983). Material bound to the column was eluted stepwise with increasing concentrations of NaCl and 2-ml fractions were collected. Glycopeptides were desalted and separated from monosaccharides by chromatography on 1  $\times$  50-cm columns of Sephadex G-25 in 7% 1-propanol. Oligosaccharides released by Endo H treatment were separated by amine adsorption high-performance liquid chromatography (HPLC) on a Beckman Model 110A dual pump system using a MicroPak AX-5 column (Varian), as previously described (Mellis and Baenziger, 1981).

#### Analysis of sugar composition of radiolabeled glycopeptides

Strong acid hydrolysis of [ $^3\text{H}$ ]mannose-labeled glycopeptides was conducted in 2 N HCl at 100 C for 4 hr. The hydrolysates were dried by evaporation under reduced pressure, resuspended in water, and analyzed by descending paper chromatography on Whatman 1

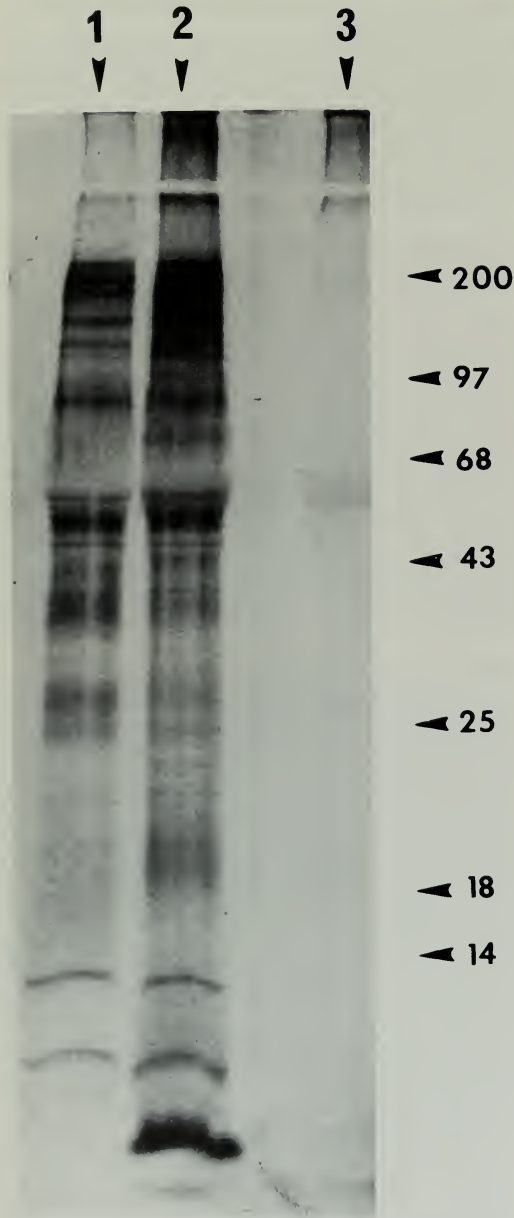


FIGURE 1. Fluorograph of SDS-PAGE of metabolically radiolabeled glycoproteins from schistosomula. Mechanically transformed schistosomula were incubated for 48 hr in media containing 4 mCi/ml of either [ $^3\text{H}$ ]mannose, [ $^3\text{H}$ ]glucosamine, or [ $^3\text{H}$ ]galactose in DMEM as described in the Materials and Methods. The radiolabeled schistosomula were solubilized and equal amounts of each homogenate were analyzed by SDS-PAGE and fluorography. Lane 1, [ $^3\text{H}$ ]mannose-labeled glycoproteins; lane 2, [ $^3\text{H}$ ]glucosamine-labeled glycoproteins; lane 3, [ $^3\text{H}$ ]galactose-labeled glycoproteins. The positions of molecular weight standards ( $\times 10^{-3}$ ) are indicated.

paper in solvent I, ethyl acetate:pyridine:water (8:2:1). [ $^3\text{H}$ ]glucosamine-labeled glycopeptides were hydrolyzed in 4 N HCl at 100 C for 4 hr. The hydrolysate was dried under reduced pressure and the released sugars were reacylated (Cummings et al., 1983) and analyzed by descending paper chromatography on borate-impregnated paper in solvent II, 1-butanol:pyridine:water (6:4:3) (Cardini and Leloir, 1957). The possible presence of radioactive sialic acid in [ $^3\text{H}$ ]glucosamine-labeled glycopeptides was assessed by mild acid treatment of glycopeptides in 2 N glacial acetic acid at 100 C for 1 hr (Cummings et al., 1983). The hydrolysate was dried under reduced pressure, resuspended in water, and analyzed by descending paper chromatography in solvent III, ethyl acetate:pyridine:acetic acid:water (5:5:1:3). N-acetylglucosaminitol, N-acetylgalactosaminitol, and oligosaccharides were separated by descending paper chromatography in solvent III. N-acetylglucosaminitol and N-acetylgalactosaminitol were also analyzed by descending paper chromatography in solvent I.

#### Release of oligosaccharides from glycopeptides by mild alkaline borohydride treatment

Radiolabeled glycopeptides were treated with 50 mM NaOH containing 1 M  $\text{NaBH}_4$  at 45 C for 16 hr (Baenziger and Kornfeld, 1974). The reaction was terminated by dropwise addition of 4 N acetic acid. Residual sodium was removed by passing the mixture over a column of Dowex 50 ( $\text{H}^+$  form). Residual borate was removed by repeated evaporation of the sample with a solution of 1 M acetic acid in methanol.

#### Glycosidase treatment

Radiolabeled glycopeptides were treated with 10 mU of Endo H in 50  $\mu\text{l}$  of 0.1 M citrate-phosphate buffer, pH 5.6, at 37 C for 48 hr in a toluene atmosphere.

## RESULTS

#### SDS-PAGE and fluorography of radiolabeled schistosomula

Mechanically transformed schistosomula were metabolically radiolabeled for 48 hr with either [ $2\text{-}^3\text{H}$ ]mannose, [ $6\text{-}^3\text{H}$ ]glucosamine, or [ $6\text{-}^3\text{H}$ ]galactose to estimate the complexity of their glycoproteins and to allow analyses of the structures of the glycoprotein oligosaccharides. Previous studies have shown that [ $2\text{-}^3\text{H}$ ]mannose is metabolized by animal cells to only radioactive mannose and fucose residues (Kornfeld et al., 1978). N-acetylglucosamine, N-acetylgalactosamine, and sialic acid residues are biosynthetically radiolabeled by [ $6\text{-}^3\text{H}$ ]glucosamine precursor (Cummings et al., 1983), and glucose and galactose residues are radiolabeled by [ $6\text{-}^3\text{H}$ ]galactose (Reitman et al., 1982; Cummings and Kornfeld, 1984). To assess incorporation of radiolabel into glycoproteins, equal numbers of radiolabeled schistosomula were solubilized and analyzed by SDS-PAGE and fluorography. Nu-



merous glycoproteins were radiolabeled by the [ $^3\text{H}$ ]mannose and [ $^3\text{H}$ ]glucosamine precursors (Fig. 1). Only 1 major glycoprotein of approximate apparent molecular weight (MW) of 60,000 was detected in the samples prepared from schistosomula incubated with [ $^3\text{H}$ ]galactose (Fig. 1).

#### Lectin affinity column chromatography of glycopeptides

Radiolabeled glycopeptides were prepared from the [ $^3\text{H}$ ]mannose- and [ $^3\text{H}$ ]glucosamine-labeled schistosomula glycoproteins by pronase digestion and applied to columns of Con A-Sepharose (Fig. 2). Unbound material was eluted with buffer alone and designated as Pool I. Bound material was first eluted with 10 mM  $\alpha$ -methylglucoside and the material eluted by the hapten was designated as Pool II. The remaining material on the column was eluted with 100 mM  $\alpha$ -methylmannoside and designated as Pool III. Most of the known O-linked oligosaccharides, complex-type bisected biantennary, tri- and tetraantennary N-linked oligosaccharides do not interact with Con A-Sepharose and may be contained in Pool I (Ogata et al., 1975; Krusius et al., 1976; Cummings and Kornfeld, 1982; Merkle and Cummings, 1987). Con A-Sepharose interacts with relatively high affinity with many of the complex-type biantennary N-linked oligosaccharides and these may be contained in Pool II (Ogata et al., 1975; Krusius et al., 1976; Cummings and Kornfeld, 1982; Merkle and Cummings, 1987). Con A-Sepharose interacts with the highest affinity with high mannose- and/or hybrid-type N-linked oligosaccharides and ungalactosylated and unsialylated complex-type biantennary N-linked chains and these may be contained in Pool III (Ogata et al., 1975; Krusius et al., 1976; Cummings and Kornfeld, 1982; Merkle and Cummings, 1987). The structures of the oligosaccharide moieties of the glycopeptides from Pools I, II, and III were analyzed by a variety of techniques.

#### Pool I glycopeptides

Analysis of radiolabeled Pool I glycopeptides by strong acid hydrolysis and descending paper chromatography indicated that the radioactivity in [ $^3\text{H}$ ]mannose-labeled Pool I glycopeptides was contained in mannose (55%) and fucose (45%); [ $^3\text{H}$ ]glucosamine-labeled Pool I glycopeptides contained N-acetylglucosamine (73%) and N-acetylgalactosamine (23%).

In our previous study on the glycoproteins synthesized by *S. mansoni* adult males, we found that the O-linked sugars were contained in Pool I glycopeptides and were released from peptide by mild base/borohydride treatment (Nyame et al., 1987). To investigate whether schistosomula also synthesize glycoproteins containing O-linked sugars, portions of the [ $^3\text{H}$ ]glucosamine-labeled Pool I glycopeptides were treated with mild base/borohydride as described in the Materials and Methods. Treatment of O-linked oligosaccharides with low concentrations of base in the presence of borohydride effects a  $\beta$ -elimination reaction, resulting in the release of the oligosaccharide from the peptide, and the borohydride catalyzes the subsequent reduction and protection of the oligosaccharides at the reducing termini (Iyer and Carlson, 1971). Under these conditions N-linked oligosaccharides are relatively insensitive to release from peptide (Debray et al., 1984; Hounsell et al., 1984). After base/borohydride treatment, the released oligosaccharides were treated to remove sodium and borate ions and then analyzed directly by descending paper chromatography in solvent III. Seventy-five percent of the radioactivity was released by base/borohydride treatment and the released sugars corresponded in migration with N-acetylglucosaminitol and N-acetylgalactosaminitol (Fig. 3). The remainder of the radioactivity was contained at the origin. The radioactive material comigrating with N-acetylglucosaminitol and N-acetylgalactosaminitol standards was eluted out of the paper and rechromatographed by descending paper chromatography in solvent I to allow more efficient separation of N-acetylglucosaminitol from N-acetylgalactosaminitol. In this system, 91% of the radioactivity comigrated with N-acetylglucosaminitol and 9% comigrated with N-acetylgalactosaminitol (Fig. 4).

Base/borohydride treatment of [ $^3\text{H}$ ]mannose-labeled Pool I glycopeptides failed to release any radioactivity migrating from the origin upon descending paper chromatography (data not shown). This suggests that the mannose and fucose residues in [ $^3\text{H}$ ]mannose-labeled Pool I glycopeptides are not in O-linked oligosaccharides and are most likely contained in complex-type N-linked oligosaccharides.

To examine the fucose linkages in Pool I glycopeptides, a portion of the [ $^3\text{H}$ ]mannose-labeled Pool I glycopeptides was applied to pea lectin-agarose. Previous studies have shown that



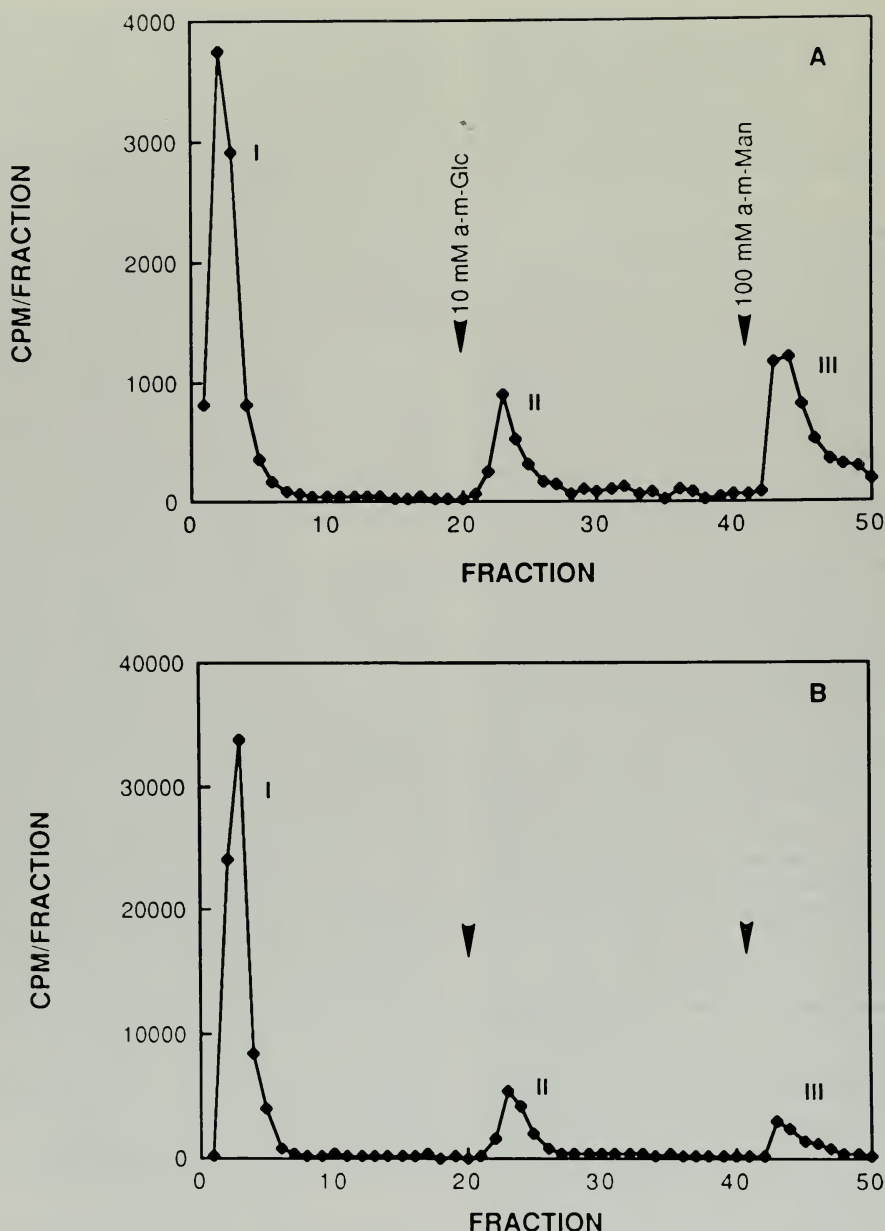


FIGURE 2. Chromatography on Con A-Sepharose of radiolabeled glycopeptides. Radiolabeled schistosomula were solubilized and treated with pronase to generate glycopeptides. The isolated glycopeptides were applied to columns of Con A-Sepharose and 2-ml fractions were collected. Bound glycopeptides were eluted sequentially with 10 mM  $\alpha$ -methylglucoside and 100 mM  $\alpha$ -methylmannoside as described in the Materials and Methods. The resulting glycopeptide fractions were pooled and designated as Pools I, II, and III as indicated. Greater than 90% of the radioactivity applied to each column was recovered. Panel A, [ $^3$ H]mannose-labeled glycopeptides; panel B, [ $^3$ H]glucosamine-labeled glycopeptides.

bi- and triantennary complex type chains that contain fucose residues linked 1,6 to the core N-acetylglucosamine residues are bound by pea lectin-agarose (Kornfeld et al., 1981; Cummings

and Kornfeld, 1982). Thirty-three percent of the radioactivity was bound by the immobilized lectin and was eluted with 500 mM  $\alpha$ -methylmannoside. The remaining radioactivity was not

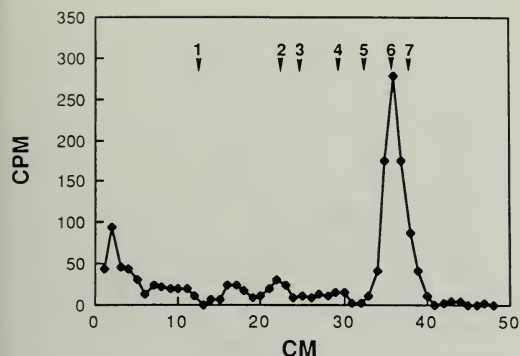


FIGURE 3. Descending paper chromatogram of [ $^3\text{H}$ ]glucosamine-labeled Pool I glycopeptides after treatment with mild base borohydride. An aliquot of [ $^3\text{H}$ ]glucosamine-labeled Pool I glycopeptides was treated with mild base/borohydride to release O-linked sugars. The sample was then treated to remove residual sodium and borate ions as described in the Materials and Methods and analyzed by descending paper chromatography in solvent III. The migration positions of standards are indicated; 1, stachyose; 2, raffinose; 3, lactose; 4, galactose 1,3N-acetylglucosaminitol; 5, galactose; 6, N-acetylglucosaminitol; and 7, N-acetylglucosaminitol.

bound by the lectin. This indicates that Pool I glycopeptides contain triantennary complex-type N-linked oligosaccharides with fucose residues attached to the core.

### Pool II glycopeptides

The sugar composition of Pool II glycopeptides was determined by hydrolyzing the glycopeptides and analyzing the released sugars by descending paper chromatography. Analysis of the [ $^3\text{H}$ ]mannose-labeled Pool II glycopeptides indicated that 67% of the radioactivity was contained in mannose and the remaining 33% was in fucose. Analysis of the [ $^3\text{H}$ ]glucosamine-labeled Pool II glycopeptides showed that it contained N-acetylglucosamine (78%) and N-acetylglucosaminitol (22%).

When a portion of the [ $^3\text{H}$ ]glucosamine-labeled Pool II glycopeptides was applied to a column of pea lectin-agarose, 23% of the radioactivity was bound by the lectin and could be eluted by 500 mM  $\alpha$ -methylmannoside. This result suggests that Pool II glycopeptides may contain complex-type biantennary N-linked chains with fucose residues attached to the N-acetylglucosamine of the inner core. In other studies, these types of glycopeptides have been found to bind to both Con A-Sepharose and pea lectin-agarose

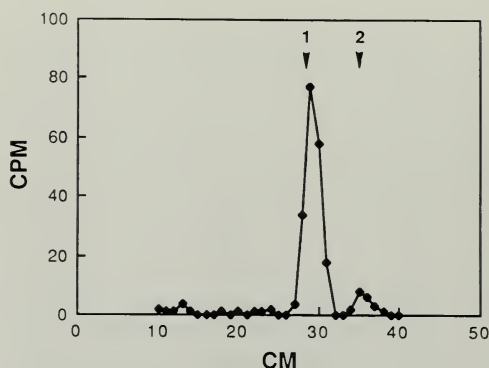


FIGURE 4. Descending paper chromatogram of N- $^3\text{H}$  acetylglucosaminitol and N- $^3\text{H}$ acetylglucosaminitol released from [ $^3\text{H}$ ]glucosamine-labeled Pool I glycopeptides by mild base/borohydride treatment. The [ $^3\text{H}$ ]glucosamine-labeled monosaccharides comigrating with the standards N-acetylglucosaminitol and N-acetylglucosaminitol in Figure 6 were analyzed by descending paper chromatography in solvent I. The migration positions of standards are indicated; 1, N-acetylglucosaminitol; 2, N-acetylglucosaminitol.

(Kornfeld et al., 1981; Cummings and Kornfeld, 1982; Merkle and Cummings, 1987).

### Pool III glycopeptides

The sugar composition of Pool III glycopeptides was determined by hydrolyzing aliquots of the [ $^3\text{H}$ ]mannose- and [ $^3\text{H}$ ]glucosamine-labeled glycopeptides and analyzing the released sugars by descending paper chromatography. All the radioactivity in the [ $^3\text{H}$ ]mannose-labeled glycopeptides was recovered as mannose. Analysis of the [ $^3\text{H}$ ]glucosamine-labeled glycopeptides indicated that 91% of the radioactivity was in N-acetylglucosamine. The remaining radioactivity was recovered as N-acetylglucosaminitol.

The composition of the Pool III glycopeptides indicated that it contained high mannose-type rather than hybrid-type N-linked oligosaccharides. To examine the structures of the high mannose-type N-linked chains, the [ $^3\text{H}$ ]mannose-labeled Pool III glycopeptides were treated with Endo H. Endo H cleaves between the 2 N-acetylglucosamine residues in the N,N-di-N-acetylchitobiose core of high mannose- and hybrid-type N-linked oligosaccharides, resulting in the release of oligosaccharides having a residue of N-acetylglucosamine at the reducing termini from GlcNAc-Asn-R (Tarentino et al., 1972; Tai et al., 1977). The Endo H-treated material was analyzed by ion-exchange chromatography on QAE-

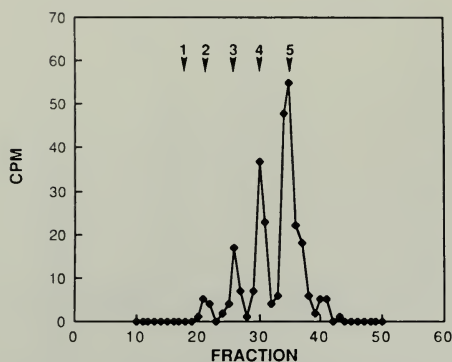


FIGURE 5. Separation by HPLC of  $[^3\text{H}]$ mannose-labeled oligosaccharides released by Endo H treatment. An aliquot of  $[^3\text{H}]$ mannose-labeled Pool III glycopeptides was treated with Endo H as described in the Materials and Methods. The neutral, released oligosaccharides were fractionated by amine adsorption HPLC on a MicroPak AX-5 column (Varian). The elution positions of standards are indicated; 1,  $\text{Man}_5\text{GlcNAc}_1$ ; 2,  $\text{Man}_6\text{GlcNAc}_1$ ; 3,  $\text{Man}_7\text{GlcNAc}_1$ ; 4,  $\text{Man}_8\text{GlcNAc}_1$ ; 5,  $\text{Man}_9\text{GlcNAc}_1$ .

Sephadex and all the radioactivity was recovered in the unbound fraction, indicating that the Endo H-released oligosaccharides were neutral. The released oligosaccharides were desalted over a column of Amberlite mixed-bed resin and the sizes of the oligosaccharides were determined by HPLC. The oligosaccharides were separated by HPLC into 4 major peaks that corresponded in elution to  $\text{Man}_6\text{GlcNAc}_1$  (3%),  $\text{Man}_7\text{GlcNAc}_1$  (9%),  $\text{Man}_8\text{GlcNAc}_1$  (20%), and  $\text{Man}_9\text{GlcNAc}_1$  (65%) (Fig. 5).

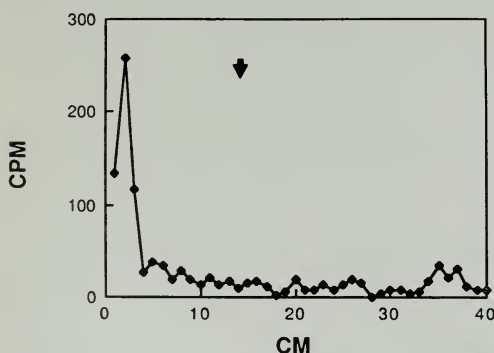


FIGURE 6. Descending paper chromatogram of  $[^3\text{H}]$ glucosamine-labeled Pool I glycopeptides after mild acid hydrolysis. An aliquot of  $[^3\text{H}]$ glucosamine-labeled Pool I glycopeptides was hydrolyzed in 2 N acetic acid to release any potential sialic acid residues and then analyzed by descending paper chromatography as described in the Materials and Methods. The migration position of the sialic acid standard is indicated.

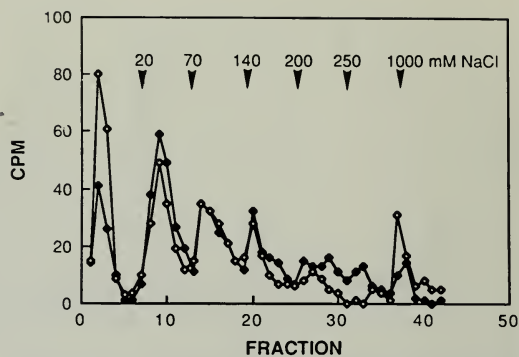


FIGURE 7. Chromatography on QAE-Sephadex of  $[^3\text{H}]$ glucosamine-labeled Pool I glycopeptides before and after mild acid hydrolysis. Aliquots of  $[^3\text{H}]$ glucosamine-labeled Pool I glycopeptides were either applied directly to a column of QAE-Sephadex or after treatment with 2 N acetic acid to release potential sialic acid residues. Bound glycopeptides were eluted with the indicated concentrations of NaCl. Recovery of radioactivity in both cases was greater than 90%.  $\blacklozenge$ , untreated glycopeptides;  $\diamond$ , mild acid-treated glycopeptides.

#### Analysis of radiolabeled glycopeptides for the possible presence of sialic acid residues

To investigate whether radioactive sialic acid was present in schistosomula glycopeptides,  $[^3\text{H}]$ glucosamine-labeled Pool I glycopeptides were treated with mild acid to release any potential sialic acid residues and then analyzed directly by descending paper chromatography. No released, radiolabeled sialic acid was detected (Fig. 6). Additionally, the  $[^3\text{H}]$ glucosamine-labeled glycopeptides showed identical elution profiles upon ion-exchange chromatography on QAE-Sephadex before and after mild acid treatment to remove any potential sialic acid residues (Fig. 7). This result indicates that sialic acid does not contribute to the anionic character of the  $[^3\text{H}]$ glucosamine-labeled glycopeptides from schistosomula.

#### DISCUSSION

This study demonstrates that *S. mansoni* schistosomula synthesize glycoproteins containing O-linked and N-linked sugar chains. The major O-linked sugars were found to be simple monosaccharides consisting of terminal O-linked N-acetylgalactosamine, the minor type, and terminal O-linked N-acetylglucosamine, the major type. Some of the N-linked oligosaccharides were found to be high mannose-type chains ranging in size from  $\text{Man}_6\text{GlcNAc}_2$  to  $\text{Man}_9\text{GlcNAc}_2$ .



Our results also suggest that schistosomula synthesize complex-type N-linked oligosaccharides.

It is interesting that many different schistosomula glycoproteins were radiolabeled metabolically by the [ $^3\text{H}$ ]mannose and [ $^3\text{H}$ ]glucosamine precursors, whereas only a single, major, schistosomula glycoprotein of approximate apparent MW of 60,000 was radiolabeled by the [ $^3\text{H}$ ]galactose precursor. Hayunga and Sumner (1986c) also found that 48-hr schistosomula incorporate [ $^3\text{H}$ ]galactose into 1 major glycoprotein of apparent MW of 43,000. In contrast, we and others have found that adult schistosomes incorporate [ $^3\text{H}$ ]galactose into numerous glycoproteins (Hayunga and Sumner, 1986b; Nyame et al., 1987). We also observed some major differences in the band pattern obtained by SDS-PAGE and fluorographic analysis of the [ $^3\text{H}$ ]mannose- and [ $^3\text{H}$ ]glucosamine-labeled glycoproteins from schistosomula and the band pattern obtained in our previous analysis of similarly radiolabeled glycoproteins from adult male schistosomes (Nyame et al., 1987). This result is consistent with the studies of others that indicate developmental regulation in the expression of *Schistosoma mansoni* glycoproteins (Payares and Simpson, 1985; Hayunga and Sumner, 1986c).

We found that glycoproteins synthesized by schistosomula lack sialic acid, which is similar to our results with glycoproteins synthesized by adult male schistosomes (Nyame et al., 1987). In addition, others have reported that no sialic acid could be detected in the tegument of adult schistosomes by direct chemical assays, whereas some sialic acid was detected in the carcasses of adult schistosomes (Robertson and Cain, 1985). In this latter study, the authors concluded that the sialylated glycoconjugates in the carcass were derived from ingested erythrocytes. It is not known whether this lack of sialylated glycoproteins is characteristic of all schistosome species. Whether sialic acid exists in the glycoconjugates of *S. mansoni* is controversial, because others have claimed its presence in tegumental glycoconjugates of some stages of the parasite. It has been reported that the binding of peanut agglutinin to lung-stage schistosomula and adult schistosomes of *S. mansoni* was enhanced after treatment of the organisms with neuraminidase, whereas there was no enhancement of binding of the lectin to newly transformed schistosomula after neuraminidase treatment (Simpson and Smithers, 1980; Simpson et al., 1983). In another

report (McDiarmid and Podesta, 1984), it was found that the binding of positively charged ruthenium red dye to the tegument of lung-stage schistosomula and adult schistosomes of *S. mansoni* was eliminated after treatment of the organisms with neuraminidase. Although these approaches using neuraminidase treatment followed by lectin or dye binding are indirect, the conclusions were made that some glycoconjugates in lung-stage schistosomula and adult schistosomes contain sialic acid, whereas glycoconjugates of newly transformed schistosomula lack it (Simpson and Smithers, 1980; Simpson et al., 1983; McDiarmid and Podesta, 1984). The use of the technique of metabolic radiolabeling in the analysis of schistosome glycoconjugates should resolve the controversy about the presence of sialic acid in other schistosome glycoconjugates, because this technique eliminates the possibility of contaminating the analyses with host-derived material.

We have shown that schistosomula synthesize glycoproteins containing high levels of O-linked N-acetylglucosamine and relatively low amounts of O-linked N-acetylgalactosamine. Previous studies indicated that adult schistosomes synthesize the 2 O-linked sugars in similar proportions on their total glycoproteins (Nyame et al., 1987). We also found that glycoproteins from schistosomula lack the O-linked disaccharide galactose-N-acetylgalactosamine, whereas this disaccharide was found to be a major component of glycoproteins synthesized by adult male schistosomes (Nyame et al., 1987). These results suggest that there are differences in the glycosylation of protein in schistosomula versus adult schistosomes.

It is possible that there is developmental regulation in either the synthesis of glycoproteins containing the O-linked GalNAc and Gal-GalNAc or the galactosyltransferases catalyzing the formation of these side chains. Our observations are consistent with some previous studies that assessed the binding of peanut agglutinin to intact schistosomes in which it was shown that schistosomula contained fewer binding sites for this lectin compared to adult schistosomes (Simpson and Smithers, 1980; Simpson et al., 1983). Peanut agglutinin has been demonstrated to bind with high affinity to the disaccharide Gal $\beta$ 1, 3GalNAc (Goldstein and Hayes, 1978).

The existence of terminal O-linked N-acetylglucosamine was first observed in some glyco-

proteins of rodent origin (Torres and Hart, 1984; Holt and Hart, 1986). The observation of this type of glycosylation in glycoproteins from adult schistosomes (Nyame et al., 1987) and schistosomula suggest that it is a common posttranslational modification of proteins. Recent studies indicate that terminal O-linked N-acetylglucosamine residues are found on glycoproteins of the nuclear pore complex in rat liver (Holt et al., 1987; Snow et al., 1987). The function(s) of these glycoproteins in mammalian cells are not yet known, although it is possible that some of these glycoproteins are involved in nuclear import through the nuclear pore (Finlay et al., 1987). The subcellular location and function(s) of schistosome glycoproteins containing terminal O-linked N-acetylglucosamine residues are not known.

The high mannose-type oligosaccharides synthesized by 48-hr schistosomula were similar in size and overall composition to those synthesized by adult male schistosomes (Nyame et al., 1988) that are also similar in structure to high mannose-type chains synthesized by mammalian cells (Nyame et al., 1988). In the present study we found that many of the oligosaccharides in glycoproteins synthesized by schistosomula have features shared by some complex-type N-linked chains of mammalian glycoproteins. These features include certain aspects of carbohydrate composition and high-affinity binding to Con A-Sepharose and pea lectin-agarose. Thus, it is possible that there are some similarities between schistosomes and mammalian cells in their processing of the N-linked oligosaccharides. However, our observation that schistosomula-derived glycopeptides contain little if any sialic acid and galactose, indicates that there are also some major structural differences in the complex-type chains from schistosomula, as compared to typical mammalian cell-derived glycoproteins.

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## HOST-PARASITE RELATIONSHIPS OF CARYOPHYLLAEID CESTODES AND AQUATIC OLIGOCHAETES: II. EFFECTS OF HOST AGE AND MIXED INFECTIONS

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**ABSTRACT:** Experiments were conducted to assess the relationship between annelid age and susceptibility of the annelid as an intermediate host for a caryophyllaeid, as well as the effect a mixed-species infection has on rate of metacestode development and parasite mortality. Four host-parasite systems were studied: *Biacetabulum biloculoides* and *Hunterella nodulosa* in *Limnodrilus hoffmeisteri* and *Glaridacris catostomi* in *Ilyodrilus templetoni* and *Tubifex tubifex*. Annelids were divided into 3 age classes: immature, approximately 7 days old; intermediate, approximately 14 days old; and mature, approximately 30 days old. Immature oligochaetes in all groups were more susceptible to infection, their parasites exhibited a lower mortality rate than 30-day-olds, and the total number of parasites in this group was higher than for the other age classes. Metacestodes reached the infective stage earlier in immature as compared to older oligochaetes. Interspecific competition between *B. biloculoides* and *H. nodulosa* resulted in increased parasite mortality and a slower rate of parasite development for the species that became established second. A prior infection with one species of cestode also affected the susceptibility of *L. hoffmeisteri* to infection with another species.

Our first report on caryophyllaeid cestode-aquatic oligochaete interactions dealt with host longevity and parasite intensity and the role these factors play in intermediate host suitability (Courtney and Christensen, 1987). The present paper addresses 2 additional features of this unique association that affect oligochaete susceptibility to infection, host age, and mixed cestode species interactions.

Age resistance by hosts to parasites is commonly seen. The relationship between host age and parasite fauna can vary depending upon the host-parasite system. Calentine (1964) observed that young oligochaetes are more easily infected with caryophyllaeid cestodes than older ones. Annelids older than 30 days are usually refractory to infection. Part of this study was designed to quantify this relationship between age and host susceptibility in terms of host infection rates and host and parasite mortality. The remainder of the study involves mixed-species infections. The vast majority of literature on natural and experimental infections of oligochaetes with caryophyllaeids involves single-species infections (Calentine, 1964; Calentine et al., 1970). The effect of a mixed infection of 2 species of caryophyllaeids on host susceptibility to infection and rate of metacestode development was investigated.

### MATERIALS AND METHODS

Three species of caryophyllaeids, *Biacetabulum biloculoides* Mackiewicz and McCrae, 1965, *Glaridacris catostomi* Cooper, 1920, and *Hunterella nodulosa* Mackiewicz and McCrae, 1962, and 3 species of tubificids, *Ilyodrilus templetoni* Southern, 1909, *Limnodrilus hoffmeisteri* Claparède, 1862, and *Tubifex tubifex* Müller, 1774, were used. All annelids used were from laboratory colonies/cultures. Two trials were done for each experiment.

Oligochaetes were experimentally infected by exposure to embryonated cestode eggs in mud-free water, under aeration for 48 hr at a temperature of 18-21°C. Both experimental and control groups were maintained under aeration in 100-ml glass beakers containing initially sterile mud and fed a diet of Tetramin® fish food. Wet mounts of infected annelids were made and examined at 100× and/or 430× with bright-field or phase-contrast optics.

### Age study

The 4 groups of cestodes and oligochaetes studied were: *B. biloculoides* and *L. hoffmeisteri*, group 1; *H. nodulosa* and *L. hoffmeisteri*, group 2; *G. catostomi* and *I. templetoni*, group 3; and *G. catostomi* and *T. tubifex*, group 4. Cocoons that contained visible worms were harvested from laboratory cultures for each species and examined regularly for hatched annelids. Oligochaetes of similar sizes were grouped together. Approximately 7, 14, and 30 days postgrouping, these annelids were exposed to cestode eggs (1,200-1,800 eggs/25 ml of water). All annelids were examined microscopically immediately after exposure to cestode eggs, and infected annelids of each age class were placed in separate beakers. Oligochaetes were categorized as immature (approximately 7 days old), no reproductive structures present; intermediate (approximately 14 days old), increase in total length and reproductive structures beginning to develop in some individuals; and mature (approximately 30 days old), testes present.

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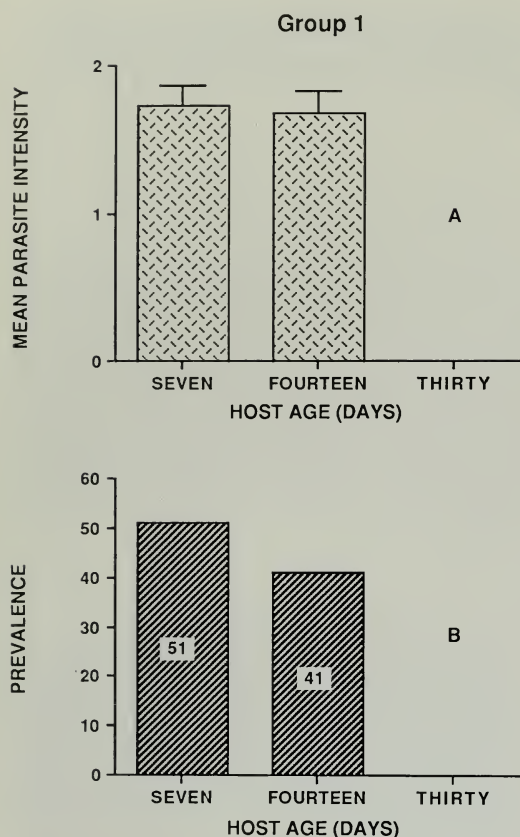


FIGURE 1. Group 1, *Biacetabulum biloculoides*-infected *Limnodrilus hoffmeisteri*. A. Mean parasite intensity for the 3 age classes after initial exposure to cestode eggs. The range bars represent the standard error of the mean. B. Prevalence of infection for the 3 age classes after initial exposure to cestode eggs. The numbers inside the bars represent the total number of infected hosts for each age class.

Annelids were examined on a weekly basis until 14 days postexposure (PE), then they were observed every 2 wk. The length of the experiment was 63 days for group 2 and 77 days for groups 1, 3, and 4. Percentage cumulative parasite mortality was determined by dividing the total number of parasites that died by the total number of parasites after initial exposure to cestode eggs for each day PE. Both prevalence of infection and total host mortality between each of the age classes were compared using  $R \times C$  contingency tables with Chi-square analysis. One-way analysis of variance was used to compare mean parasite intensities between the 3 age classes. Tukey's studentized range test was used to determine which means were different from each other. Student's *t*-test was used when only 2 sample means were compared. Differences in each of these analyses were considered significant at  $P < 0.05$ .

#### Mixed infection study

Only *L. hoffmeisteri*, *B. biloculoides*, and *H. nodulosa* were used in these experiments. Annelids were

divided into 4 groups and exposed to 1,000–1,500 eggs/25 ml of water. *Limnodrilus hoffmeisteri* in group 1 were first exposed to *H. nodulosa* eggs, then infected individuals were exposed to *B. biloculoides* eggs 14 days later. Group 2 oligochaetes (controls for group 1) were not exposed to *H. nodulosa*, but were exposed to *B. biloculoides* at the same time as group 1. The third group of *L. hoffmeisteri* was first exposed to *B. biloculoides* eggs and infected individuals were exposed to *H. nodulosa* eggs 14 days later. Group 4 annelids (controls for group 3) were not exposed to *B. biloculoides*, but were exposed to *H. nodulosa* at the same time as the third group. The experiment was ended after 80 days following the first exposure to caryophyllaeids. Student's *t*-test was used to assess differences between the mean parasite intensity of experimental groups and their corresponding control group. Prevalence was compared using  $R \times C$  contingency tables with Chi-square analysis. Differences were considered significant at  $P < 0.05$ .

Aquatic oligochaetes acquire their caryophyllaeid parasites through the ingestion of the infective egg. Inside the gut, the oncosphere hatches out of the egg and bores through the intestinal wall into the coelom. Depending on the species of cestode, it may complete its development here to the infective metacystode stage or in the seminal vesicles of the host. Both *B. biloculoides* and *H. nodulosa* develop in the latter location in *L. hoffmeisteri*, although some individuals in both of these cestode species remain in the coelom. *Limnodrilus hoffmeisteri* possesses anterior and posterior seminal vesicles. The former is produced by the forward extension of the septal wall between segments 9 and 10 and the latter is a result of the pouching of the septal wall between segments 10 and 11. This vesicle containing the parasite increases in size and extends posteriorly with the growth of the tapeworm (Calentine, 1963). Infective metacystodes are generally considered infective for the definitive host when they possess a highly differentiated scolex, a cercomer, and primordia of gonads. Only the latter 2 characteristics were used for recognizing *H. nodulosa* metacystodes, because they do not possess a highly differentiated scolex.

## RESULTS

### Age study

Prevalence and mean parasite intensity decreased after initial exposure to cestode eggs (Figs. 1–4). Significant differences were found between the prevalence of infection in each of the 4 cestode–annelid groups examined.

Figure 5 shows mean parasite intensity at weekly intervals postexposure (PE) for all systems. Significant differences between the means at the different days PE were noted only in group 4 (Fig. 5D). In this group, mean parasite intensity at day 1 PE was significantly greater than means at days 14 through 77 for immature *T. tubifex* infected with *G. catostomi*.

No significant differences were seen when mean parasite intensity was compared between 7- and



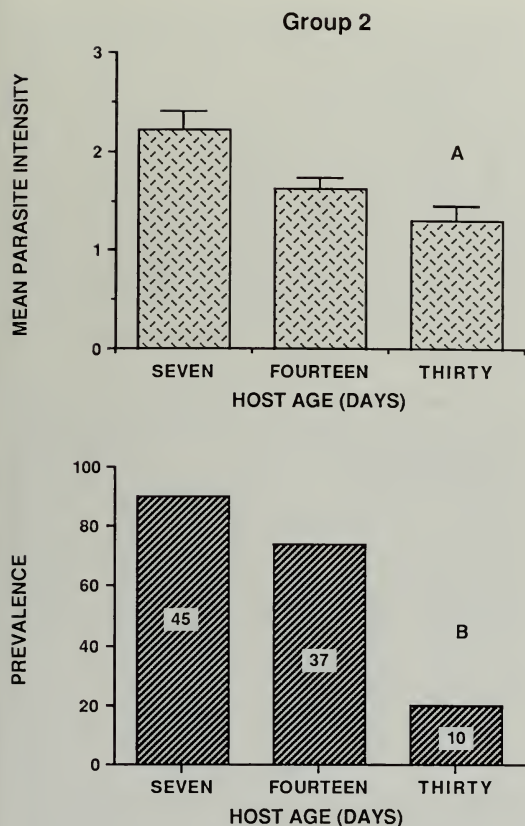


FIGURE 2. Group 2, *Hunterella nodulosa*-infected *Limnodrilus hoffmeisteri*. A. Mean parasite intensity for the 3 age classes after initial exposure to cestode eggs. The range bars represent the standard error of the mean. B. Prevalence of infection for the 3 age classes after initial exposure to cestode eggs. The numbers inside the bars represent the total number of infected hosts for each age class.

14-day-old *L. hoffmeisteri* infected with *B. biloculoides* for each day PE (Fig. 5A). Only 1 30-day-old *L. hoffmeisteri* became infected, and this individual harbored only 1 cestode. At 63 days PE, 90% (19/21) of the parasite population was at the infective stage in immature annelids and 38% (6/16) in 14-day-old hosts. Overall, parasite mortality was similar for metacestodes in 7- and 14-day-old *L. hoffmeisteri* (Fig. 6A), even though almost 50% of the parasites were dead in 14-day-old hosts at 7 days PE compared to 20% in immature annelids. Parasite mortality figures represent parasite death that resulted both from host death and hosts that lost their infection.

Mean parasite intensity for 7-day-old *H. nodulosa*-infected *L. hoffmeisteri* was significantly higher than for 14- and 30-day-olds at 1 day PE

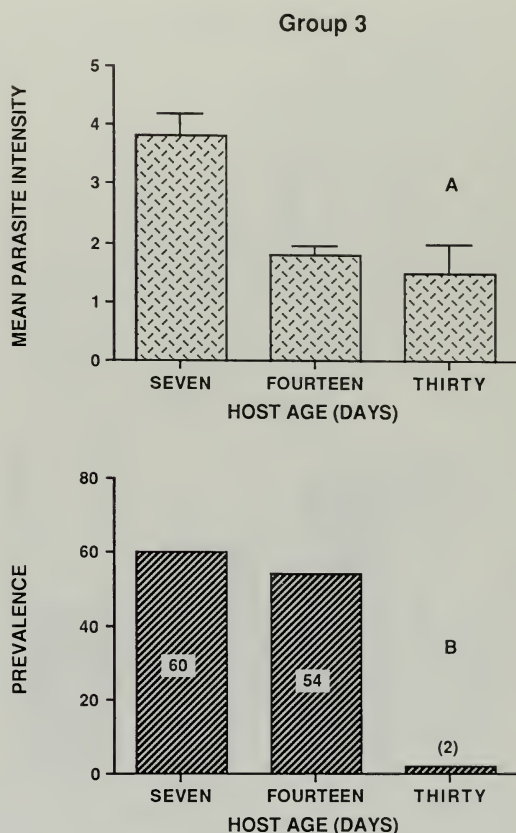


FIGURE 3. Group 3, *Glaridacris catostomi*-infected *Ilyodrilus templetoni*. A. Mean parasite intensity for the 3 age classes after initial exposure to cestode eggs. The range bars represent the standard error of the mean. B. Prevalence of infection for the 3 age classes after initial exposure to cestode eggs. The numbers inside the bars and in parentheses represent the total number of infected hosts for each age class.

(Fig. 5B). Significant differences also were seen between the means at 28 and 63 days PE for 7- and 14-day-old annelids. Rate of cestode mortality increased with the age of the host (Fig. 6B). All mature *L. hoffmeisteri* lost their infection by 28 days PE (Figs. 5B and 6B). At 63 days PE, 100% (54/54) and 75% (15/20) of the *H. nodulosa* were at the infective stage in the 7- and 14-day-old *L. hoffmeisteri*, respectively.

Only 2 mature *I. templetoni* became infected and harbored a total of 3 *G. catostomi* in group 3, and both individuals lost their infections by 7 days PE (Fig. 5C). Significant differences were found between mean intensity at day 1 for all 3 age groups. Significant differences were found when mean parasite intensities were compared between 7- and 14-day-old annelids at days 1

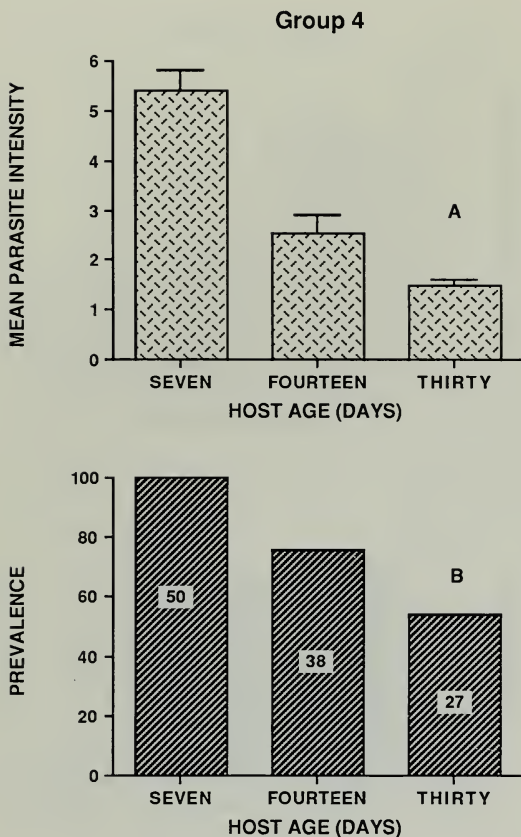


FIGURE 4. Group 4, *Glaridacris catostomi*-infected *Tubifex tubifex*. A. Mean parasite intensity for the 3 age classes after initial exposure to cestode eggs. The range bars represent the standard error of the mean. B. Prevalence of infection for the 3 age classes after initial exposure to cestode eggs. The numbers inside the bars represent the total number of infected hosts for each age class.

through 28 PE. There was little difference in the rate of cestode mortality in 7-day-old compared with 14-day-old *I. templetoni* (Fig. 6C), but only *G. catostomi* infecting immature *I. templetoni* reached the infective stage, 34% (15/44), by the end of the experiment.

In group 4 (Fig. 5D), significant differences were

found between mean parasite intensity for the 3 age groups at days 1 and 7. Again, mean parasite intensity was significantly higher in the 7-day-old oligochaetes. By 28 days PE, all 30-day-old annelids had lost their infections (Figs. 5D and 6D). Mature annelids exhibited the highest percentages of parasite mortality and differences in cestode mortality between 7- and 14-day-old annelids were greatest at 14 through 42 days PE (Fig. 6D). Thirty percent (7/23) of *G. catostomi* in the 7-day-old *T. tubifex* and 18% (3/17) in 14-day-old hosts were at the infective stage at 77 days PE.

For each host-parasite system studied, differences between total host mortality for the various age classes were not significant. Mature annelids possessed the lowest mortality rates (Table I). A greater percentage of 14-day-old hosts lost their infection compared to 7-day-old annelids in all groups (Table II). Highest percentage of hosts losing their infection occurred in 30-day-old annelids.

#### Mixed infection study

Mean parasite intensity and prevalence of *B. biloculoides* in *H. nodulosa*-infected *L. hoffmeisteri*, group 1, and the controls, group 2, are shown in Table III. A total of 115 *L. hoffmeisteri* containing 155 *H. nodulosa* was present at the beginning of the experiment. Sixty-six of these annelids were still infected at 14 days PE and were exposed to *B. biloculoides*. Mean parasite intensity was significantly different for *B. biloculoides* in mixed and single infections (controls) at 2 and 10 days PE. Large differences between the percentage of hosts losing their *B. biloculoides* cestodes were seen for single (33%) and mixed (71%) infections. No significant differences were found between the prevalence on initial exposure to *B. biloculoides* for *H. nodulosa*-infected (32%) and control *L. hoffmeisteri* (43%). In mixed infections, *B. biloculoides* (38%) were first found in the seminal vesicle of the host at 48 days PE,

TABLE I. Percent total host mortality at 63 days postexposure (PE) for group 2 and 77 days PE for groups 1, 3, and 4.

Host-parasite system (group)	Percentage host mortality		
	7 days old	14 days old	30 days old
<i>B. biloculoides</i> and <i>L. hoffmeisteri</i> (1)	33 (17/51)*	29 (12/41)	
<i>H. nodulosa</i> and <i>L. hoffmeisteri</i> (2)	18 (8/45)	32 (12/37)	10 (1/10)
<i>G. catostomi</i> and <i>I. templetoni</i> (3)	38 (23/60)	30 (16/54)	
<i>G. catostomi</i> and <i>T. tubifex</i> (4)	40 (20/50)	29 (11/38)	26 (7/27)

\* Total number of hosts dead divided by total number of infected hosts at the beginning of the experiment.



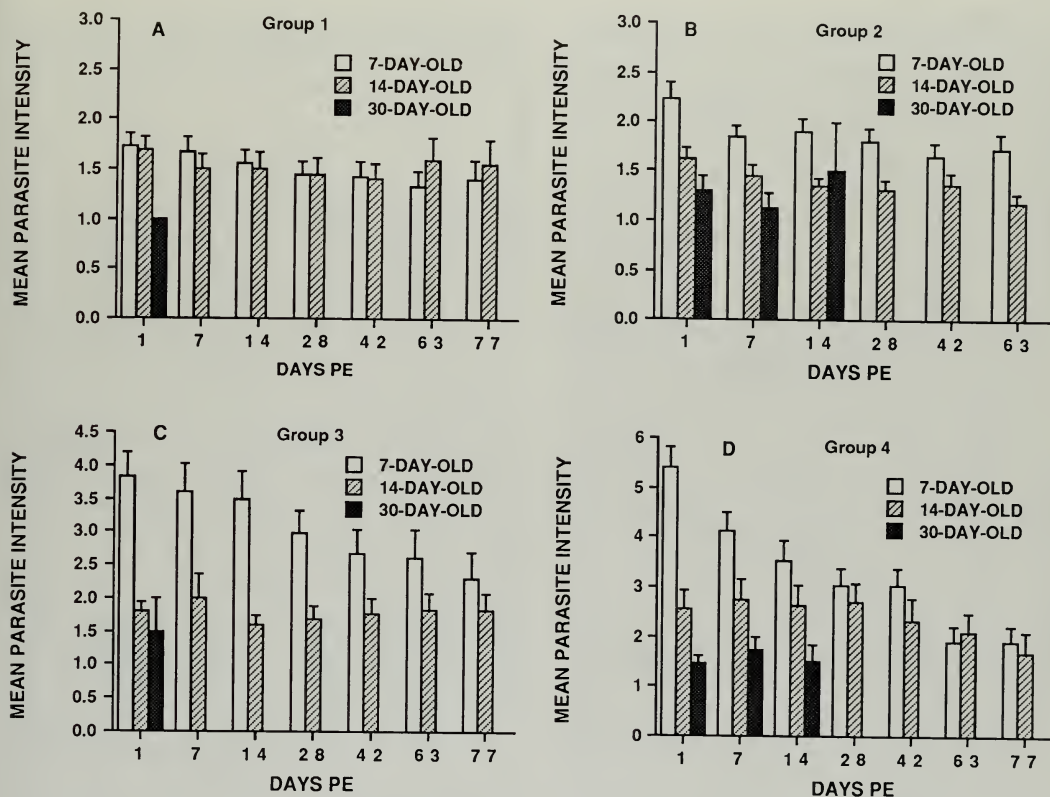


FIGURE 5. Mean parasite intensity at various time intervals for the 3 age classes in the 4 caryophyllaeid-annelid systems studied. The range bars represent the standard error of the mean. A. Group 1, *Biocetabulum biloculoides*-infected *Limnodrilus hoffmeisteri*. B. Group 2, *Hunterella nodulosa*-infected *L. hoffmeisteri*. C. Group 3, *Gliridacris catostomi*-infected *Ilyodrilus templetoni*. D. Group 4, *G. catostomi*-infected *Tubifex tubifex*.

however the procercoids did not reach the infective stage by 64 days PE. Metacystodes of *B. biloculoides* in single infections were first observed in the seminal vesicle at 33 days PE, and by 48 days PE all cestodes were in the seminal vesicle with 41% (7/17) at the infective stage. Significant differences were found between parasite mortality in single and mixed infections of *B. biloculoides*, at 10 and 64 days PE, 2% vs. 36% and 60% vs. 91%, respectively. All *H. nodulosa* metacystodes in the mixed infections were in-

fective and in seminal vesicles at 64 days PE. *Hunterella nodulosa* in single infections were first seen in seminal vesicles at 26 days PE. At 64 days PE, 97% (28/29) were at the infective stage.

Mean parasite intensity and prevalence of *H. nodulosa* in *B. biloculoides*-infected *L. hoffmeisteri*, group 3, and controls, group 4, are presented in Table III. A total of 86 *L. hoffmeisteri* containing 161 *B. biloculoides* was present initially. Sixty-five of these oligochaetes were still infected at 14 days PE and were exposed to *H.*

TABLE II. Percent total hosts that lost their infection at 63 days postexposure (PE) for group 2 and 77 days PE for groups 1, 3, and 4.

Host-parasite system (group)	Percentage losing infection		
	7 days old	14 days old	30 days old
<i>B. biloculoides</i> and <i>L. hoffmeisteri</i> (1)	39 (20/51)*	49 (20/41)	
<i>H. nodulosa</i> and <i>L. hoffmeisteri</i> (2)	13 (6/45)	22 (8/37)	90 (9/10)
<i>G. catostomi</i> and <i>I. templetoni</i> (3)	32 (19/60)	54 (29/54)	
<i>G. catostomi</i> and <i>T. tubifex</i> (4)	20 (10/50)	45 (17/38)	74 (20/27)

\* Total number of hosts that lost their infection divided by total number of infected hosts at the beginning of the experiment.



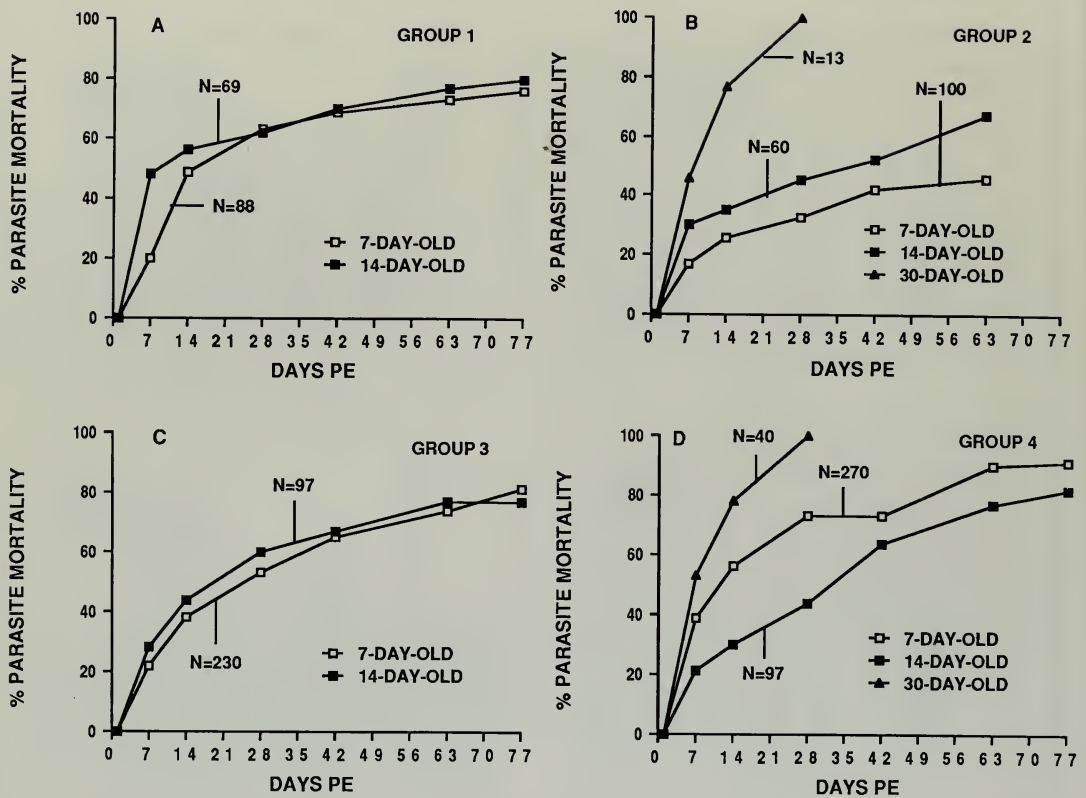


FIGURE 6. Percent parasite mortality at various days postexposure (PE) for each age class in the 4 caryophyllaeid-annelid systems studied. Sample sizes correspond to the total number of parasites at the beginning of the experiment. A. Group 1, *B. biloculoides*-infected *L. hoffmeisteri*. B. Group 2, *H. nodulosa*-infected *L. hoffmeisteri*. C. Group 3, *G. catostomi*-infected *I. templetoni*. D. Group 4, *G. catostomi*-infected *T. tubifex*.

*nodulosa*. A significant difference was found between the prevalence on initial exposure to *H. nodulosa* for *B. biloculoides*-infected *L. hoffmeisteri* (9%), group 3, and the control *L. hoffmeisteri* (71%), group 4. None of the hosts harboring mixed infections lost their *H. nodulosa* cestodes; however, 45% of the hosts containing single infections did. Metacystodes of *H. nodulosa* in both single and mixed infections were first

observed in seminal vesicles at 33 days PE. All were infective at 64 days PE in the former and none became infective in the latter. In both single and mixed infections, *B. biloculoides* metacystodes were first seen in the seminal vesicle at 26 days. At 49 days PE, 26% (12/47) of the metacystodes in single infections and 100% (14/14) of those in mixed infections were at the infective stage. Significant differences between parasite

TABLE III. Prevalence, mean parasite intensity, and percent host losing infection in *Limnodrilus hoffmeisteri* harboring single and mixed infections of *Hunterella nodulosa* and *Biacetabulum biloculoides*.

Group	Initial infection	Subsequent infection	% Prevalence 2 days PE*	Mean parasite intensity (SE)			% Hosts losing infection 64 days PE
				2 days PE	10 days PE	64 days PE	
1	<i>H. nodulosa</i>	<i>B. biloculoides</i>	32 (21/66)**	1.1 (0.1)	1.0 (0.0)	1.0 (0.0)	71
2	Controls for group 1	<i>B. biloculoides</i>	43 (30/70)	1.4 (0.1)	1.4 (0.1)	1.0 (0.0)	33
3	<i>B. biloculoides</i>	<i>H. nodulosa</i>	9 (6/65)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	0
4	Controls for group 3	<i>H. nodulosa</i>	71 (47/66)	1.2 (0.1)	1.1 (0.0)	1.0 (0.0)	45

\* PE = postexposure for subsequent infection.  
 \*\* No. infected/no. exposed.

mortality in single and mixed infections of *B. biloculoides* were seen at 49 and 64 days PE, 13% vs. 53% and 13% vs. 67%, respectively.

## DISCUSSION

In our present studies we found that annelid age has a significant effect on its susceptibility to infection with caryophyllaeids and on rate of development of the metacestodes. Immature rather than mature annelids were more successful hosts for these cestodes in all host-parasite systems studied. Initial prevalence and mean parasite intensity were highest in 7-day-old hosts. Metacestodes reached the infective stage earlier in immature annelids, and parasite mortality was lower in this age class compared to older annelids.

As the age of the host increased, susceptibility of annelids to infection decreased and a greater percentage of the older hosts lost their infection. This trend was evident in both 14-day-olds (all groups) and in groups 2 and 4 for mature annelids (*H. nodulosa*-infected *L. hoffmeisteri* and *G. catostomi*-infected *T. tubifex*). Mature oligochaetes usually did not become infected, and those that did lost their infections within a few weeks. This suggests the older annelids may be more immunologically competent or less compatible physiologically to the parasite.

Our previous studies with *B. biloculoides* and *L. hoffmeisteri* showed that this caryophyllaeid can cause considerable mortality in the host population (Courtney and Christensen, 1987). In the present study, this mortality appeared to be independent of host age because both host and parasite death rates were very similar for 7- and 14-day-old oligochaetes. It also is evident from previous studies (Courtney and Christensen, 1987) and the present study that the host-parasite association of *H. nodulosa* and *L. hoffmeisteri* is very good in immature annelids. However, in older hosts (14 days) a greater percentage of the parasite population died as a result of an increase in the number of hosts losing their infections.

Temperature is an important factor that can affect the rate of parasite development or influence the seasonal changes in population densities of parasites. When temporal and spatial distribution of host (intermediate and definitive) and parasite are controlled by this factor it can increase the probability of contact between the 2 (Esch, 1983). Jarroll (1980) found a positive correlation between egg-producing adults of *Both-*

*riocephalus rarus* in its definitive host and seasonal peaks in the abundance of the intermediate host, *Macrocylops ater*. This copepod achieved peak density in July, which coincided with maximum water temperatures. Developmental time for eggs of *B. rarus* decreases with increasing water temperature. Both these events result in an increase in the probability of contact between the intermediate host and the tapeworm.

Many species of adult caryophyllaeids undergo seasonal maturation with egg release coinciding with the availability of intermediate hosts. Kennedy (1969) showed a positive relationship between egg release by *Caryophyllaeus laticeps* and the presence of the new generation of its intermediate host, *Psammoryctes barbatus*. This oligochaete has a 1-yr life cycle with the new generation appearing in the summer (June and July). Both prevalence and intensity of the tapeworm increase during this time. As the annelids in the population age further, infection and reinfection does not occur. This applies to both infected and uninfected oligochaetes. Both Kennedy's study and ours give support to the assertion that age can determine the suitability of an oligochaete as an intermediate host for caryophyllaeids. This phenomenon is greatly influenced by temperature, which can synchronize availability of cestode eggs to young oligochaetes.

Reports of competition among tapeworm species based on experimental studies mainly involve vertebrate species (Chandler, 1939; Holmes, 1962; Ghazal and Avery, 1974; Befus, 1975). One of the few studies of mixed species interactions of parasites in an aquatic invertebrate host is that conducted by Awachie (1967). When the amphipod *Gammarus pulex* is infected with both *Polymorphus minutus* and *Echinorhynchus truttae*, these 2 acanthocephalans exhibit a slower rate of development than that seen in single species infection. Neither parasite enhances or decreases susceptibility of infection to the other.

Not only did our study show that mixed-species infections influence cestode development, but other aspects of the association as well. An initial infection with one species of caryophyllaeid significantly influenced subsequent infection with another species of caryophyllaeid. The presence of *B. biloculoides* decreased susceptibility of *L. hoffmeisteri* to infection with *H. nodulosa*. However, if *H. nodulosa* became established first, this did not affect the initial prevalence of *B. biloculoides*. No significant differences between initial



prevalence of *B. biloculoides* in single and mixed infections were seen.

A higher mortality rate of the tapeworm species in the subsequent infection was not always seen. Although mortality was lower for *H. nodulosa* when it was the initial infection compared to *B. biloculoides* as the subsequent infection, the reverse situation was not true. Overall, parasite mortality was greater for both species in mixed infections than when either species was found alone.

In mixed infections, metacestodes that became established second had a slower rate of development than those in single infections. None of these reached the infective stage by the end of the experiment. For those caryophyllaeids that became established first, a subsequent infection with another species of cestode did not have a significant effect on the rate of development.

It is apparent that both age and the presence of another species of caryophyllaeid can significantly affect the ability of an annelid to function as a suitable intermediate host. Older oligochaetes are less susceptible to infection and those that do become infected have a greater tendency to lose their infections. Interspecific competition can have a number of detrimental consequences for the tapeworm species that becomes established second, including increased parasite mortality and slower rate of development that increases the time for the metacestodes to reach the infective stage. This failure of the second caryophyllaeid species in mixed infections to achieve the infective stage could be attributed to several factors. The initial infection may stimulate the immune system of the host to such an extent that subsequent infections are destroyed, thus reducing the parasite burden, and/or the first cestode species may deplete the nutritional resources available, thereby resulting in parasite death or a slower rate of development of the second caryophyllaeid species.

Infection with *B. biloculoides* in *L. hoffmeisteri* confers resistance to susceptibility to infection with *H. nodulosa*. Whether *L. hoffmeisteri* would react similarly to a second infection with *B. biloculoides* is not known. Initial infection with *H. nodulosa* did not have a significant effect on susceptibility of this oligochaete to *B. biloculoides*. This may be related to the pathogenic nature of *B. biloculoides* in *L. hoffmeisteri*. Infection by this species causes substantial mortality in the *L. hoffmeisteri* population (Courtney and Christensen, 1987). The presence of this caryophyllaeid

may activate the immune system causing the host to respond more strongly to a subsequent infection with *H. nodulosa*. *Hunterella nodulosa* has a very good relationship with *L. hoffmeisteri* that results in high survival rates for both parasite and host. This caryophyllaeid might not activate the immune system to any great extent and consequently, susceptibility to infection with *B. biloculoides* would not be affected.

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## SPOROZOITE-INDUCED INFECTIONS OF THE SALVADOR I STRAIN OF *PLASMODIUM VIVAX* IN *SAIMIRI SCIUREUS BOLIVIENSIS* MONKEYS

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**ABSTRACT:** Twenty *Saimiri sciureus boliviensis* monkeys from Bolivia were inoculated intravenously with sporozoites of the Salvador I strain of *Plasmodium vivax*. All animals were splenectomized 7 days after inoculation. Inoculation of 100,000 sporozoites resulted in prepatent periods averaging 16.6 days; all monkeys developed high-level parasitemias with an average maximum of 103,000 per mm<sup>3</sup>. Inoculation of 10,000 sporozoites resulted in average prepatent periods of 19.4 days; one of the resulting infections produced a transient low-level parasitemia. Of 5 monkeys inoculated with 1,000 sporozoites, 4 had prepatent periods of from 24 to 35 days and 1 failed to demonstrate any parasitemia; 1 monkey supported a low-level transient parasitemia, whereas the other 3 monkeys had high-level parasitemias. It is proposed that by using a minimum inoculum of 10,000 sporozoites, the model system may be useful in the testing of anti-sporozoite vaccines directed against *P. vivax*.

Previously, we reported on our attempts to establish the Chesson strain of *Plasmodium vivax* in *Saimiri sciureus boliviensis* monkeys from Bolivia as a model for the testing of anti-sporozoite vaccines (Collins et al., 1987). Although the rate of sporozoite-induced infection was relatively high (78%), the barely detectable parasitemias during the first 50 days following inoculation indicated that this model had limited application. A good model for the testing of sporozoite vaccines would be a strain of the parasite that readily infects mosquitoes and a species of monkey that, following inoculation of sporozoites, would exhibit a high rate of susceptibility (preferably 100%) with predictable prepatent periods.

Further attempts were therefore made to develop a suitable model for the testing of sporozoite vaccines directed against *P. vivax*. Campbell et al. (1983) had reported the transmission via sporozoite inoculation of the Salvador I (Sal I) strain of *P. vivax* to *Saimiri* monkeys from Guyana, Peru, and Bolivia. One monkey from Bolivia had a prepatent period of 21 days, and supported an infection with a maximum parasitemia greater than 200,000 per mm<sup>3</sup>. Based on this observation, this model was used for additional studies, the results of which are reported here.

### MATERIALS AND METHODS

The Sal I strain of *P. vivax* was originally isolated from an infected human in the area of Cangrejera in the Department of La Paz, El Salvador (Collins et al., 1972). This strain had been maintained in *Aotus* monkeys prior to its passage to *Saimiri* monkeys.

The *S. sciureus boliviensis* originated in Bolivia and were obtained through the Agency for International Development; they were maintained in quarantine until used in the study under space conditions as set forth by the National Institutes of Health. All monkeys were fed a diet shown to provide adequate nutrition for captive *Saimiri* used in malaria-related research—this includes New World monkey chow, fresh fruit and vegetables, vitamins and minerals, and high caloric supplements, when deemed necessary by the attending veterinarian. Prior examination indicated that the animals were free of natural malarial infection.

*Anopheles stephensi* mosquitoes were infected by feeding through parafilm membranes on blood from chimpanzees infected with the Sal I strain of *P. vivax* (Collins et al., 1986). Mosquitoes were held until dissected in a constant temperature incubator at 25 ± 1 C. The procedures used for the feeding, handling, and dissection of the mosquitoes have been previously reported (Collins et al., 1966). For inoculation, salivary glands containing sporozoites were dissected from anesthetized mosquitoes into 20% fetal calf serum/0.85% saline. After being crushed under a coverslip, the sporozoites were washed into a vial and counted in a Neubauer cell-counting chamber. Suspensions containing different concentrations of sporozoites were then injected intravenously into the animals.

Seven days after inoculation, all animals were splenectomized to increase their ability to support blood-stage infections that might arise from the rupturing exoerythrocytic stages. Beginning 10–14 days after sporozoite inoculation, thick (Earle and Perez, 1932) and thin blood films were made daily and stained with Giemsa. Parasite counts were recorded per mm<sup>3</sup> of

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TABLE I. *Prepatent periods and maximum parasitemias in 10 Saimiri sciureus boliviensis monkeys inoculated with 100,000 sporozoites of the Sal I strain of Plasmodium vivax.*

Monkey	Pre-patent period	First day parasitemia >1,000/mm <sup>3</sup>	Peak parasitemia		Mosquito infection	
			Per mm <sup>3</sup>	Day*	Lots positive/fed	Percent
SS-64	14	21	103,000	47	35/36	97.2
SS-55	14	28	105,000	54	29/30	96.7
SS-70	15	25	142,000	52	23/27	85.2
SS-67	15	23	188,000	53	13/25	52.0
SS-66	16	35	125,000	52	24/25	96.0
SS-53	17	32	74,200	52	25/27	92.6
SS-65	17	27	106,000	49	26/29	89.7
SS-57	18	36	36,600	46	7/16	43.8
SS-63	18	32	104,000	53	11/18	61.1
SS-71	22	37	49,500	47	11/15	73.3
Mean	16.6	29.6	103,000	50.5		

\* Days after sporozoite inoculation.

blood. To determine if the animals were supporting the development of infective gametocytes, mosquitoes were fed on those monkeys that developed parasitemia greater than 1,000 per mm<sup>3</sup>. At the end of the study, all animals were administered 30 mg chloroquine (base) by oral intubation over a period of 3 days and 2.5 mg of primaquine daily for 7 days.

*Anopheles freeborni*, originally from Marysville, California, have been maintained continuously in our laboratory since 1944. *Anopheles stephensi* (STE-2 strain), originally from Delhi, India, were obtained from Dr. Robert W. Gwadz, National Institutes of Health, and have been maintained in our laboratory for several years.

## RESULTS

A total of 20 monkeys were used in the study. In the first experiment, 10 monkeys were inoculated, each with approximately 100,000 spo-

rozoites obtained by salivary gland dissection from infected *An. stephensi*. Prepatent periods ranged from 14 to 22 days with a mean of 16.6 days (Table I). Maximum parasitemias, which averaged 103,000 per mm<sup>3</sup>, were obtained between 46 and 54 days after inoculation; these highest parasite counts occurred between the 24th and 39th days of patent parasitemia. Parasite counts in the monkeys inoculated with the Sal I strain reached the 1,000 per mm<sup>3</sup> level on an average of 29.6 days after inoculation (after an average of 13.0 days of patent parasitemia).

Based on our previous results with the feeding of mosquitoes on New World monkeys infected with *P. vivax*, which indicated that mosquito infection may occur early in the infection, mosquito feeding was begun at or near the time when the parasite count reached the 1,000 per mm<sup>3</sup> level. A total of 248 mosquito lots were fed, 199 lots of *An. freeborni* and 49 lots of *An. stephensi*. The percentage of mosquito lots infected is shown in Table I. Overall, 82.3% of the lots contained infected mosquitoes. The intensity of gut infections in *An. freeborni* was higher than in *An. stephensi*, but the percentages of infected mosquitoes were very similar.

To further determine the usefulness of this model, 10 additional monkeys were inoculated with sporozoites dissected from the salivary glands of *An. stephensi* mosquitoes infected by feeding on blood from an infected chimpanzee. In this experiment (Table II), 5 monkeys were inoculated, each with an estimated 10,000 sporozoites and 5 monkeys were inoculated each with 1,000 sporozoites. All monkeys inoculated with 10,000 sporozoites developed patent par-

TABLE II. *Prepatent periods and maximum parasitemias in Saimiri sciureus boliviensis monkeys inoculated with 10,000 or 1,000 sporozoites of the Sal I strain of Plasmodium vivax.*

Monkey	Sporozoite inoculum	Prepatent period	First day parasitemia >1,000/mm <sup>3</sup>	Peak parasitemia		Mosquito infection	
				Per mm <sup>3</sup>	Day*	Lots positive/fed	Percent
SI-28	10,000	15	28	53,600	50	25/35	71.4
SI-16	10,000	16	32	163,000	58	29/34	85.6
SI-168	10,000	21	31	108,000	52	27/33	81.8
SI-171	10,000	22	—	62	25	—	—
SI-197	10,000	23	38	16,000	44	12/15	80.0
Mean		19.4	32.3	68,100	45.8		
SI-224	1,000	24	—	217	36	—	—
SI-225	1,000	24	46	50,800	70	16/17	94.1
SI-219	1,000	30	36	54,300	60	25/27	92.6
SI-294	1,000	35	43	139,000	67	20/21	95.2
SI-283	1,000	—	—	—	—	—	—
Mean		28.3	41.7	61,100	58.3		

\* Days after sporozoite inoculation.



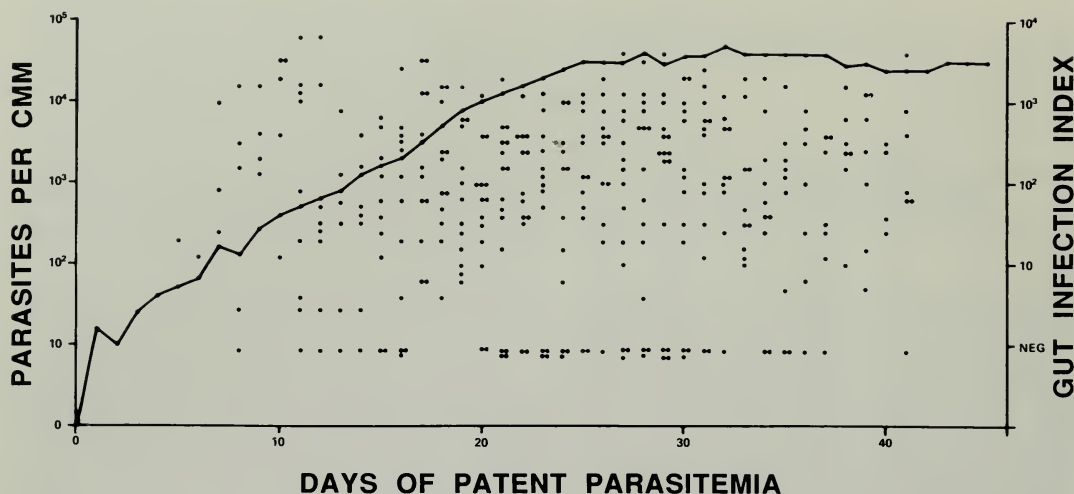


FIGURE 1. Mean parasitemia and mosquito infection levels during the first 45 days of patent parasitemia for 17 *Saimiri sciureus boliviensis* monkeys infected via sporozoite inoculation with the Sal I strain of *Plasmodium vivax*. Solid line = mean parasitemia; dots = individual gut infection indexes (gut infection index = average number of oocysts per 100 guts).

asitemias between 15 and 23 days (mean 19.4 days). Four of the 5 monkeys developed high-level parasitemias with maximum counts occurring between 44 and 58 days after inoculation. One monkey (SI-171) developed a transient parasitemia with a maximum count of 62 per  $\text{mm}^3$ .

Four of the 5 monkeys inoculated with 1,000 sporozoites developed patent parasitemias after periods of 24–35 days (mean 28.3 days). One animal, SI-283, did not develop a detectable parasitemia and monkey SI-224 had a low-level transient infection with a maximum parasitemia of 217 per  $\text{mm}^3$ . Maximum parasitemias occurred in the 3 monkeys with high-level parasite counts 60–70 days after sporozoite inoculation.

Mosquitoes were fed on the 7 monkeys that developed high-level parasitemias. Because the courses of parasitemia in the 17 monkeys that developed high-level parasite counts were similar, the results of the mosquito feedings on these animals were combined (Fig. 1). Results are presented only for the *An. freeborni* feedings through day 45 of patent parasitemia. Results were presented according to the day of patent parasitemia rather than according to the days after sporozoite inoculation, because the prepatent periods were extended in the animals receiving fewer sporozoites; once parasites were detected, the course of the parasitemia was similar. As shown, high-level mosquito infection was obtained from day 11 through day 45 with little variation. Of 352 lots of mosquitoes fed and dissected, 84.5% were

infected. On 55 occasions, lots of mosquitoes had gut infection indexes (average number of oocysts per 100 guts) of greater than 1,000. Within the lots, the percentage of infected mosquitoes varied little from day to day, although there was a greater variation in the average number of oocysts per infected gut. Some animals were more heavily infected, and some animals had shorter periods of high-level infection either followed or preceded by low-level or negative days.

## DISCUSSION

The Sal I strain of *P. vivax* readily infected *S. sciureus boliviensis* following the intravenous inoculation of sporozoites dissected from the salivary glands of infected *An. stephensi* mosquitoes. The prepatent periods were dose related, with the earliest prepatent periods obtained following the inoculation of 100,000 sporozoites. Based on these results, it is proposed that the Sal I strain of *P. vivax* and the Bolivian *S. sciureus boliviensis* monkey be used as a model for the testing of sporozoite vaccines. Because there is a dose-related response to the sporozoite inoculum with regard to length of the prepatent period and the development of detectable parasitemia, a minimum of 10,000 sporozoites should be used for challenge studies.

At the lower levels of sporozoite inocula, 2 animals, SI-171 and SI-224, developed detectable parasitemias with prepatent periods in the range of the other animals. The ensuing levels of

parasitemia, however, were low and transient. This is difficult to understand because, once a blood-stage infection is induced, the sporozoite inoculum should have little or no effect on the course of the parasitemia. Whether the cause lies with these particular host animals or is actually an effect of the smaller number of sporozoites inoculated remains to be shown. It is possible that the merozoites released from the liver may have differing capabilities of developing in these hosts. With an inoculation of 100,000 sporozoites, all animals received parasites that, upon release, were capable of inducing high-level infections. With fewer numbers of sporozoites introduced, these more virulent forms may be eliminated, and only the parasites capable of producing transient infections may remain. These latter parasites would probably be present in the larger inocula, but their presence would be masked by that of the more virulent parasites. Because 1,000 sporozoites failed to produce an infection in monkey SI-283, it is possible that either many of the sporozoites are noninfective or that once within the host, they are somehow prevented from migrating to the liver or from completing their development. In addition, in this abnormal host, many of the merozoites released from the liver may not be capable of establishing a blood-stage infection. Thus, it can be speculated that 4 types of sporozoites were introduced into the host—one type that can produce a highly virulent infection, another a transient infection, a third incapable of producing a detectable parasitemia, and a fourth that enters a resting stage (hypnozoite) that eventually may cause relapse. However, extensive additional study would be required to support this explanation. Whatever the reason, the number of sporozoites required to induce high-level or even transient infections is much greater than is probably needed to induce a human infection. It points out that New World monkeys, although very useful for a number of malarial studies with the human infecting species, are often dissimilar from humans in many respects concerning infection and transmission of plasmodia. This fact must be taken into account in interpreting many of the results of transmission and immunization studies.

It is apparent that the *S. sciureus boliviensis*

monkey and the Salvador I strain of *P. vivax* have significant potential for the testing and development of anti-sporozoite vaccines. In addition, mosquito infection was readily obtained from all 17 of the monkeys that developed significant parasitemias. Mosquito infectivity studies indicated that these animals can produce infective gametocytes for many days. Thus, in addition to serving in vaccine trials, this model can readily be used for production of both blood-stage parasites and sporozoites for immunologic and serologic studies.

#### ACKNOWLEDGMENTS

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## EXPERIMENTAL TRANSMISSION, DEVELOPMENT, AND EFFECTS OF A PARASITIC COPEPOD, *LERNAEOCERA BRANCHIALIS*, ON ATLANTIC COD, *GADUS MORHUA*

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**ABSTRACT:** Development and effects of adult *Lernaocera branchialis* were studied following experimental transmission to Atlantic cod, *Gadus morhua*. Growth from the detection of pennella stages to mature adults was approximately 9-10 mo (September-June of the following year) at which time eggs were released and the adult parasites degenerated. Cod from all size groups were susceptible but the prevalence of infection was greater in small fish. Peak mortality, about 30%, occurred within 4 mo after infection and was greatest in young fish with multiple infections. Death was associated with emaciation, blood loss, open lesions, and probable occlusion of branchial blood vessels and/or ventral aorta. Infected fish, especially subadults that survived, consumed less food, gained less weight, had a lower conversion factor, and were substantially smaller than uninfected controls through a 32-wk period. Many of these fish displayed hyperactivity. Large cod that survived the infection and harboured 1 parasite were apparently unaffected but died when stressed. A previous infection conferred no protection against reinfection. Multiple infections delayed gonadal maturation and resulted in significantly lower gonadal somatic indices than in controls. Exposure of infected fish to crude oil fractions or to infection with a hemoprotozoan, *Trypanosoma murmanensis*, culminated in mortality, weight loss, or low organ somatic indices. It is estimated that considerable losses through mortality and weight gain in young fish occur each year in coastal areas from infections by *L. branchialis*, particularly in one area where it was estimated that 20% of the population was infected.

*Lernaocera branchialis* is a hematophagous copepod in its adult phase, infecting several species of gadoid fishes (Kabata, 1970). It induces anemia, loss of weight, reduces fat content and reproductive capacity, and is believed to cause mortality (Stekhoven, 1936a; Kabata, 1958; Mann, 1970; Van den Broek, 1978; Hislop and Shanks, 1979). Kabata (1984) recently reviewed various aspects of the parasite's biology and remarked that several areas required clarification. Although Stekhoven (1936b) illustrated developmental stages of the adult phase on gadoid fish, the rate of development and longevity are still unknown. The present study, initiated in the summer of 1984, investigated the development and duration of the parasite's cycle on gadoids and its effects on growth and mortality. In addition, experiments were conducted to assess the effect of 2 concurrent stressors, viz. water-soluble fractions of a crude oil and a hemoprotozoan parasite, *Trypanosoma murmanensis*, on cod harbouring *L. branchialis*.

### MATERIALS AND METHODS

Atlantic cod, 32-49 cm in length, were trapped and held in a flow-through seawater system in 3,000-L

aquaria or in a concrete raceway 700 × 250 × 100 cm (flow rate, 50 L/min) for 6 wk to a year prior to infection (vide Khan and Lacey, 1986). The fish were fed capelin, *Mallotus villosus*, 2-3 times weekly. Common lumpfish, *Cyclopterus lumpus*, that are naturally infected with larval stages of *L. branchialis* (cf. Templeman et al., 1976) were caught in the month of July 1984-1986 by SCUBA divers and placed in tanks with uninfected cod. Control cod were kept in separate tanks or a raceway. Sampling for *L. branchialis* infection, following transmission, was performed at intervals thereafter. A number of infected and uninfected cod were tagged and held in tanks to compare feeding and growth rates more accurately. The length and weight of each fish as well as the number and location of specimens of *L. branchialis* that were present were determined at intervals after infection. Hemoglobin values were ascertained occasionally. The parasites were classified subsequently according to the scheme as outlined by Sproston and Hartley (1941) and Evans et al. (1983). A record was also made of lesions caused by parasites after detachment. Gill colour of each fish that succumbed was classified as red, pink, or pale (Evans et al., 1983). The latter, almost white, has been used as indicator of anemia (Slinn, 1970). At necropsy, the length and body and organ weights were determined. Blood was also removed and hemoglobin and packed cell volumes determined. Condition factor ( $W/L^3$ ) and organ somatic indices (percent organ weight/total body weight) were determined from the last-mentioned variables. Thin blood films were prepared, fixed with methanol, stained with Giemsa, and examined for cellular changes. Experiments were also carried out to ascertain food conversion (percent weight gained/food consumed) in control and infected subadult cod. The fish were fed, to satiation, chopped capelin, which was weighed prior

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TABLE I. A comparison of condition factor (KF), liver somatic index (LSI), and hemoglobin (Hb) values in Atlantic cod that succumbed within 4 mo after infection with *L. branchialis* and uninfected controls.

Maturity	Cod group	No. of fish	$\bar{x}$ total length	Variable ( $\bar{x} \pm \text{SE}$ )		
				KF ( $\times 100$ )*	LSI	Hb
Subadult	Control	12	40 $\pm$ 1	85 $\pm$ 3	6.2 $\pm$ 0.5	5.8 $\pm$ 0.2
	Infected (a)†	11	37 $\pm$ 2	62 $\pm$ 2‡	4.1 $\pm$ 0.5‡	2.1 $\pm$ 0.1‡
	Infected (b)	11	36 $\pm$ 1	52 $\pm$ 3‡	2.9 $\pm$ 0.4‡	2.0 $\pm$ 0.2‡
Adult	Control	10	55 $\pm$ 2	77 $\pm$ 3	7.3 $\pm$ 0.6	8.2 $\pm$ 0.5
	Infected	10	58 $\pm$ 2	67 $\pm$ 12	3.6 $\pm$ 0.9‡	2.4 $\pm$ 0.4‡

\* Based on carcass weight.

† Group (a) died within 1–2 mo and group (b) at 3–4 mo after infection.

‡ Significantly different from controls ( $P < 0.05$ ).

to feeding. The weight of each cod was also determined at the conclusion of each experiment. In experiments involving the effects of crude oil fractions as a stressor on infected cod, the latter and control (uninfected) fish were exposed to water-accommodated oil fractions of a Venezuelan crude in a 3,000-L flow-through tank (vide Khan and Kiceniuk, 1984). Eight subadult cod, 35–40 cm in length, were exposed to oil fractions (100  $\mu\text{g/L}$ ), 7 wk after experimental infection with *L. branchialis*, for 6 wk and subsequently removed and held for an additional 6 wk. Adult fish, 48–54 cm in length, were treated with a higher concentration of water-accommodated hydrocarbons (500  $\mu\text{g/L}$ ) for 4 wk. These fish also had been infected experimentally 10 mo previously with *L. branchialis*. An equal number of corresponding parasite-free fish were also treated with oil fractions, whereas 2 untreated groups, control and parasitized, were held under oil-free conditions until necropsy. Total hydrocarbon concentrations in seawater were determined by gas chromatography as reported previously (Khan and Kiceniuk, 1984).

When the effect of a trypanosome, *Trypanosoma murmanensis*, was studied in cod parasitized by *L. branchialis* 4 mo following experimental infection, a protocol similar to that used previously was followed (Khan and Lacey, 1986). Seventeen *L. branchialis*-infected and an equal number of parasite-free cod were infected with *T. murmanensis* via leeches harbouring infective stages, and held for 12 wk. Corresponding control fish were held separately, and all fish were fed to satiation at the ambient water temperature ( $\sim 2^\circ\text{C}$ ) until necropsy, when the lengths and weights were determined.

The data were analysed by a 2-way ANOVA for significant differences within each group with the respective controls. In addition, the Duncan multiple range test was used to separate groups that were significantly different. Means and standard errors were determined for all values.

## RESULTS

### Initial observations

A female lumpfish (34 cm), harbouring larval stages of *L. branchialis*, was placed in a tank (3,000 L) with 15 cod (38–55 cm) in July 1984. When the fish were examined in the latter part

of October, all harboured 1–3 *L. branchialis*. Between October 1984 and April 1985, 6 cod (38–46 cm) succumbed, each harbouring 2–3 parasites. Only fish with single infections survived. Deaths were associated with pale gills, low hemoglobin values ( $< 2.0 \text{ g/100 ml}$ ), and emaciation. Egg strings were first observed in mid-December as the parasites increased in size, attaining their maximum size in May–June 1985. Examination of these fish again in October, December, and in April 1986 revealed a progressive degeneration of the main body of the parasite from dark red to grey with disappearance of the egg strings. Finally, only a thin transparent membranous covering remained attached to a stalk, which eventually dropped off, leaving a stub. Only the latter was observed in 6 cod that survived to June 1986, approximately 2 yr after infection.

In July 1985, a group of 25 cod were held with 2 lumpfish, harbouring larval stages of *L. branchialis*, in order to confirm the previous observations. In mid-August, 10 cod were removed into a separate tank from the lumpfish and replaced by another 10 tagged fish of comparable size (36–49 cm). An additional 7 tagged cod were placed in the same tank in mid-September. A group of 50 control cod were held in 2 adjacent tanks without lumpfish. Twenty-nine of 32 cod present in the tanks in July and August with the lumpfish became infected. The 10 cod that were removed in mid-August, the 7 cod introduced in mid-September, and the 50 control fish did not acquire infections of *L. branchialis*. Seven of the 29 cod that acquired the infection harboured a single parasite, whereas the remaining 22 fish contained 2–12 parasites. Fifty-nine percent (17) of the infected fish (30–52 cm) died at intervals between October and April 1986. Eighty-two percent (14) of the deaths occurred within 4 mo after the infections were detected initially. Again,

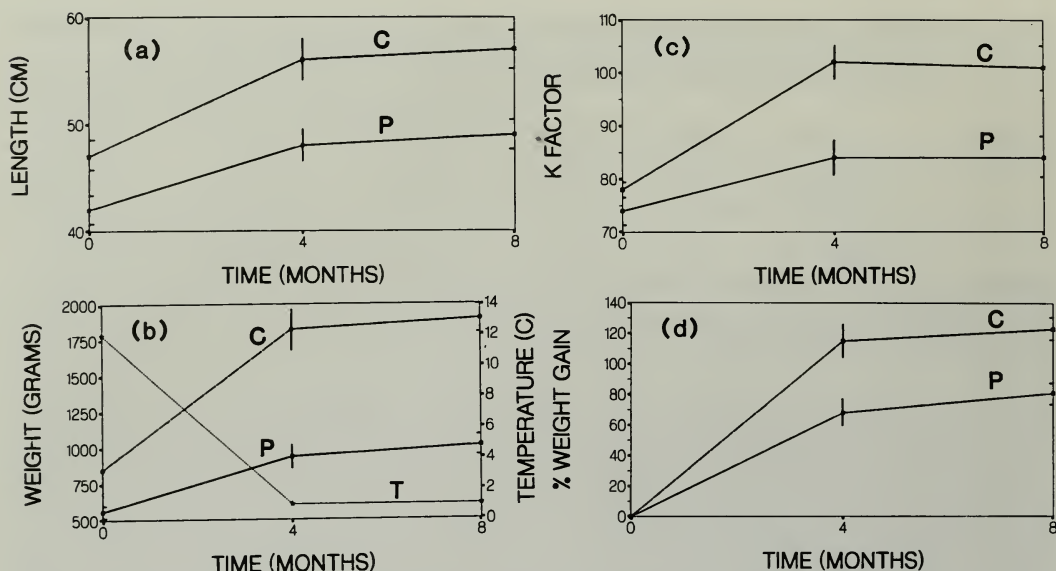


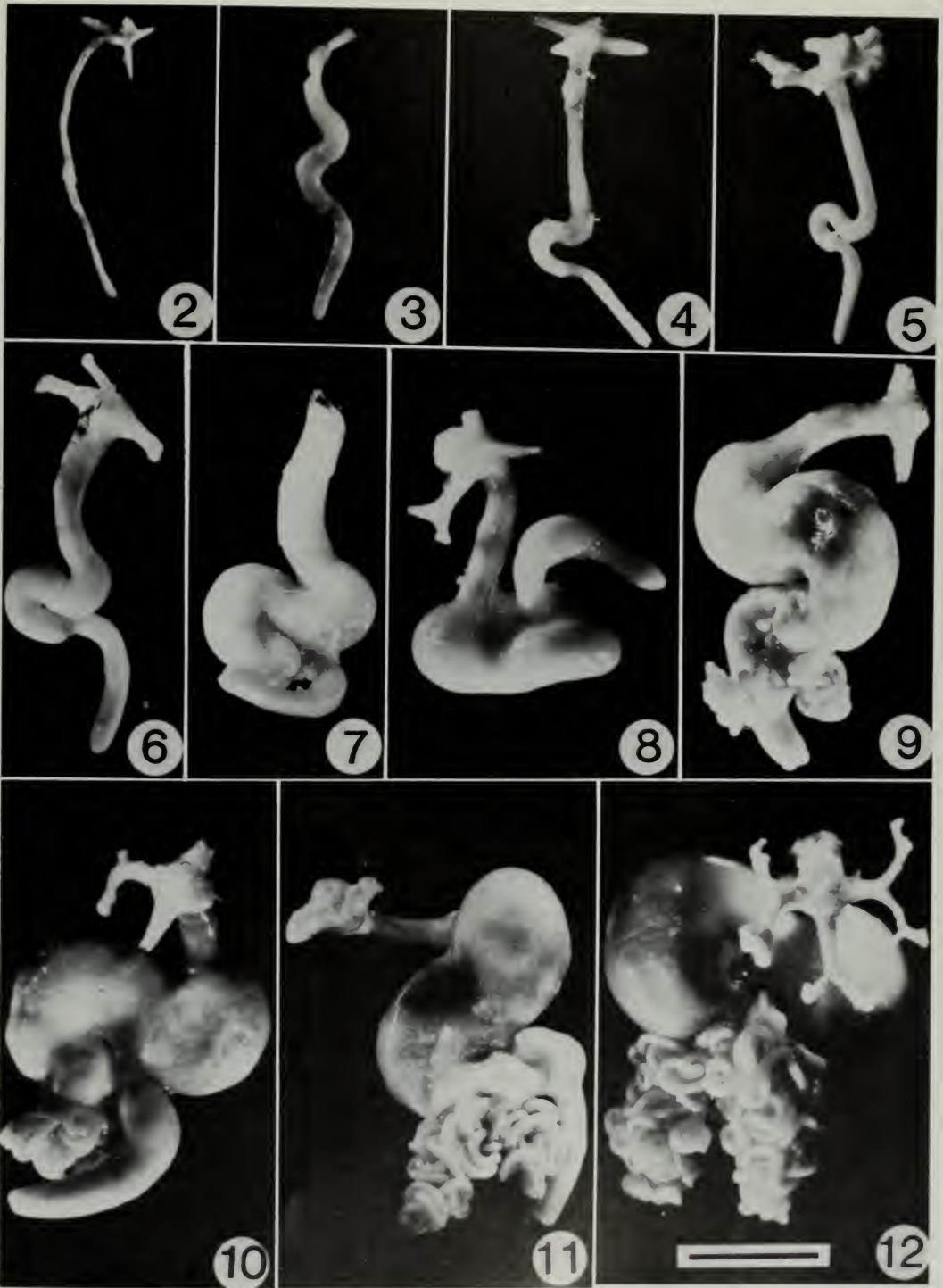
FIGURE 1. Comparison of mean total body length, weight, k-factor, and percent weight gain in control ( $n = 10$ ) and infected cod ( $n = 10$ ) parasitized by *L. branchialis* over an 8-mo period (1985–1986). C = control, P = parasitized cod. Deviations are SE. A typical seawater temperature (T) profile between September and April (month 0–8) is shown in Fig. 1b.

deaths were associated with multiple infections, pale gills, low hemoglobin levels ( $<2.0$  g/100 ml), low liver somatic indices, emaciation, and low k-factor (Table I). Among the remaining infected fish that survived, weight gain was consistently lower (10% after 4 mo and 35% after 8 mo) than among controls (Fig. 1).

Development of the parasite was followed more closely commencing from the early pennella stages to mature adults (Figs. 2–13). Young parasites (Fig. 2), measuring 11–16 mm, were pale pink and were recognized only with difficulty among the gill filaments. However, as growth was accompanied with flexure (Figs. 3–5), they were readily recognized as typical lernaecoceran copepods, each with a dark red trunk. This was described as a post-pennella stage (“U”) by Sproston and Hartley (1941). Specimens taken from fish necropsied in mid-September displayed a considerable increase in size. The trunk flexed (U-shaped) and 3 antlers appeared at the anterior extremity (Figs. 3, 5). The parasites were considerably larger during October (Fig. 6), and

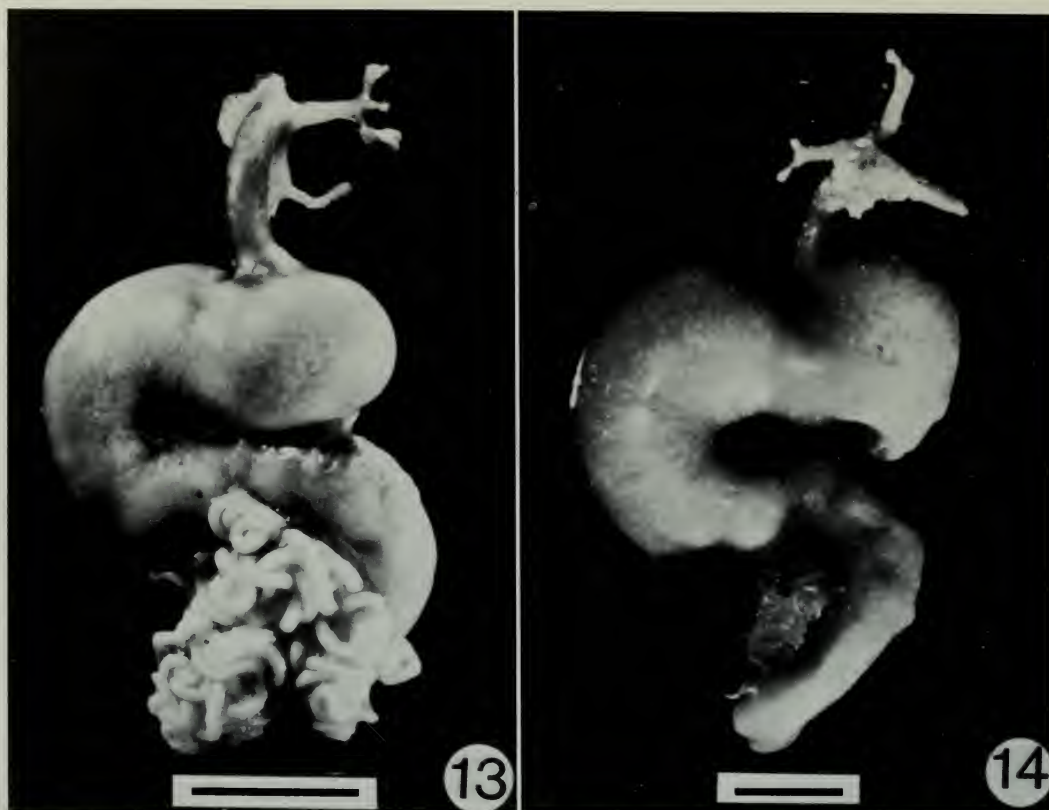
by November the trunk acquired a bulky and compact appearance (“W” stage) (Fig. 7). At least 1 of the antlers of the cephalothorax displayed dichotomy (Fig. 8). Egg sacs (“X” stage) were first observed during December in 1 of 10 specimens (Fig. 9). However, the number of individuals with egg sacs increased to 7 of the 10 examined during the month of January (Fig. 10), and no specimens were observed without egg strings after the month of March. The egg sacs increased in size with time (Figs. 11, 12). Initially, the cephalothorax of the parasite extended along the branchial artery, increased in length, and eventually acquired several antlers. The egg mass attained its maximum size during June (Fig. 13). During September, about 1 yr after infection, the parasite’s body assumed a dark grey color as the egg strings decreased in size and became darker (Fig. 14). Eventually, no egg strings were observed and only a transparent cuticle with a stalk remained attached to the branchial region. The percentage of stalks increased from 25% (6 of 18) in September to 50% (9 of

FIGURES 2–12. Development of *L. branchialis* on Atlantic cod. Scale bar is 0.5 cm. 2. Pennella (‘P’) stage; young form with almost straight body recently attached to the gill of cod (September 3–1 wk). 3. Young (‘U’) form, displaying torsion (September 14–2 wk). 4. Similar ‘U’ form, about 1 wk later (September 22–3 wk).



5. Later 'U' form (September 29—4 wk). 6. Late 'U' form (October 14—6 wk). 7. Early 'W' form (October 30—8 wk, anterior extremity inadvertently decapitated). 8. Genital segment is apparent ('W') form (November 16—10 wk). 9. Parasite with egg strings ('x' stage B, December 12—14 wk). 10–12. 'X' stages with increasing egg string size taken in January 1986 (19 wk), February (22 wk), and April 1986 (8 mo), respectively.





FIGURES 13, 14. Development of *L. branchialis* on Atlantic cod. Scale bar is 0.5 cm. 13. Fully developed female (June–10 mo). 14. Fully developed female with spent egg strings ('y' stage, October–13 mo).

18) by November and to 72% (13 of 18) by the last week in January 1987. Two specimens persisted without egg strings until summer, i.e., about 10 mo after the eggs were released.

Examination of Giemsa-stained blood smears prepared from fish with multiple infections revealed erythrocytic changes. A predominance of polychromatic erythrocytes and fewer erythroblasts were observed in fish in which pale gills and low hemoglobin ( $<4.0$  g/100 ml) and hematocrit ( $<12\%$ ) values were recorded. In many smears, there was evidence of "smudge" (ruptured) cells. The latter appeared, on the basis of staining intensity and partially ruptured cells, to be mainly early polychromatic erythrocytes.

Appearance of infections of *L. branchialis* occurred over a short period of time in the third year of study (1986) among a group of cod held with an infected lumpfish from mid-July to mid-September. Infections were not observed following necropsies of 12 cod from August 10 to 18 inclusive, but the pennella stage was noted in 1 of 9 fish examined between August 19 and 26,

1986. However, from August 28 to September 5, 6 of 9 cod necropsied harboured parasites, and among another group of cod examined but not necropsied on September 10, 10 of 12 were infected with *L. branchialis*. Development of the parasites was similar to that reported in the 1985–1986 group. However, among a group of 12 cod that were retained in seawater 2 C higher than the ambient temperature (6–10 C, vide Fig. 1b), egg strings appeared about a month earlier (November) than in the other groups.

#### Prevalence, mortality, and intensity

To ascertain prevalence and intensity, 271 cod were exposed and about 73% (198) acquired infections with *L. branchialis* in 1986 (Table II). Prevalence appeared to be greatest (95%) among the smallest size class (29–35 cm) and it decreased with increasing length. Mortality corresponded to some degree with prevalence as it was greatest (60% of 55) among juvenile fish ( $\leq 41$  cm). Approximately 71% (39) of 55 cod that succumbed in the first 4 mo were  $\leq 44$  cm in length.

TABLE II. Prevalence and mortality caused by *L. branchialis* in different size classes of Atlantic cod within 8 mo following infection.

Size class (cm)	Total exposed	No. infected	% Infected	No. died	% Died (no. died/no. infected)
29–35	41	39	95	15	38
36–41	58	46	80	20	43
42–47	53	38	72	12	32
48–53	45	30	67	10	33
≥54	74	45	61	14	31
Total	271	198	73	71	36

Forty-nine percent (35) of the deaths occurred in the first 2 mo following infection (Table III), 77% in the first 4 mo and fewer in the succeeding 4 mo.

Intensity of the infection varied from 1 to as many as 12 parasites per host (Table IV). About 31% harboured 1 parasite but more harboured multiple infections. Mortality was greater (40–53%) in fish harbouring multiple infections than among the group infected with 1 parasite (18%). Of 39 cod (≥44 cm) that died within 4 mo following infection, 84% (33) harboured 2 or more parasites.

Death in the first 2 mo following infection was associated with multiple infections and open, hemorrhagic lesions (1–2 mm in diameter) in the branchial chamber after some parasites were shed. Severe necrosis, characterized by a yellow caseous mass, was observed, especially in the second month (October) after infection, near the point of attachment of each parasite. This was more severe especially when bilateral infections were present on opposite sides of the branchial chamber between the fourth and fifth branchial arches. The musculature was discoloured, fragile, and necrotic. About 20% of the deaths were associated with this type of infection. Additionally,

TABLE III. Death distribution from 1 to 8 mo in cod infected with *L. branchialis*.

Month after infection	No. died	% Died
1	11	15
2	24	34
3	13	18
4	7	10
5	5	7
6	3	4
7	4	6
8	4	6
Total	71	100

TABLE IV. Intensity of *L. branchialis* and mortality in Atlantic cod (29–73 cm) 1–8 mo after infection.

No. of parasites	No. of fish infected	% Infected of total infected	No. died after infection	% Died after infection
1	62	31	11	18
2	45	23	18	40
3	38	19	20	53
≥4	53	27	22	42
Total	198	100	71	36

when parasites attached to the proximal part of the gill arch, the filaments distal to the point of attachment were blanched. The remaining gills were also pale and hemoglobin values were low (<2.0 g/100 ml). In fish (≤44 cm) with multiple infections, there was a tendency for some parasites to be shed, leaving an open lesion. This either became necrotic, with a caseous exudate or healed completely, leaving an area that was slightly elevated. Healing occurred more rapidly in adults especially in spring as there was little or no evidence of scars when these were examined 5 wk later. Ninety percent of the parasites attached to the area adjacent to the ventral aorta especially when a single parasite was present. When any of these fish died unexpectedly, antlers of the parasite were located either in the ventral aorta or the branchial artery. On no occasion did parasites attach to the heart (bulbus arteriosus). However, some did attach to unusual sites such as the inner wall of the operculum (4) and fewer (2) to the tongue.

Infections by *L. branchialis* were also associated with hyperactivity, erratic swimming, and a tendency from some fish, especially within 3 mo after infection, to remain at the surface. These fish acquired an intense, dark pigmentation and invariably were unable to swim and feed properly. The gills were pink, and both hemoglobin ( $\bar{x}$ ,  $2.2 \pm 0.1$ ) and hematocrit ( $\bar{x}$ ,  $12 \pm 2$ ) values were low. In 2 groups of infected fish, about 10% displayed these symptoms. Among 39 cod (≤44 cm) that were autopsied, the swim bladder was distended in 18% (15) of 55 fish in 1986. This was not observed in uninfected cod that were necropsied during the third month (1986) of the experiment.

#### Reduction in intensity

There was evidence of a reduction in the intensity of parasitism with time in cod infected with *L. branchialis*. In an initial study, it was



TABLE V. *Decrease in intensity of L. branchialis infecting cod at 4 and 8 mo after infection.*

Variable	Sampling time (mo)*	
	4	8
No. of fish infected with $\geq 2$ parasites/total no. infected	46/92 (50)†	43/78 (55)‡
$\bar{x}$ parasite intensity (92 and 78 fish, respectively)	2.2 $\pm$ 0.2	2.0 $\pm$ 0.2
No. of fish losing parasites/total infected	22/92 (24)	13/78 (17)
$\bar{x}$ no. of parasites shed/fish	1.7 $\pm$ 0.1	1.2 $\pm$ 0.1
No. of parasites shed/total no. of parasites	33/202 (16)	16/158 (10)
No. of fish becoming parasite free/total no. of infected fish	4/92 (4)	3/78 (4)
No. of fish that reduced parasite burden to 1/total no. of multi-infected fish	8/46 (17)	6/43 (14)
No. of fish that reduced parasite burden to 2/no. infected with $\geq 3$ parasites	7/35 (20)	7/31 (23)

\* Infected/total (%).

† Data were obtained for the same group of fish that were held in a raceway.

‡ Discrepancy between values taken at 4 and 8 mo are the result of fish that succumbed after 4 mo and are not included at 8 mo.

observed that 6 of 17 subadult cod ( $\leq 44$  cm in length) shed from 1 to 2 parasites in the third month (November) following infection. Also, among 18 adult fish (48–64 cm), 6 shed 1 parasite when multiple (2–4) infections were present, whereas in the other 12 additional hosts infected with 1 parasite none shed.

To ascertain the extent of parasitic reduction more precisely, a group of 92 and subsequently 78 cod were examined at 4 and 8 mo, respectively, after infection with *L. branchialis*. Seventeen to 24% of these fish shed mean numbers of 1.2–1.7 parasites/fish when the mean intensity of parasitism varied from 2.0 to 2.2 parasites (Table V). Although 10–16% of the total number of parasites were shed, only 4% of the fish became eventually free of parasites. The intensity of parasitism decreased by 14–17% from 2 or more parasites to 1, whereas 20–23% reduced their burden from 3 or more to 2 parasites per fish host. The overall reduction per group was 0.36 and 0.21 at 4 and 8 mo, respectively.

#### Susceptibility to reinfection

A study was conducted to ascertain whether or not cod, harbouring previous infections, could be reinfected. Thirty-five adult cod ( $\bar{x}$  length, 61  $\pm$  3.2 cm) that had been infected in the laboratory in the previous year, were placed in raceways containing a lumpfish harbouring devel-

opmental stages of *L. branchialis*. Thirty-one uninfected cod of comparable length were also placed in the same tank. Sixty-three percent (22) of the 35 cod harbouring previous infections ( $\bar{x}$ , 1.64  $\pm$  0.21 parasites/host) became infected with additional parasites (2.59  $\pm$  0.37), whereas 55% (17) of the 31 cod in the uninfected group acquired 1.94  $\pm$  0.41 parasites per host. However, these apparent differences between the 2 groups were not statistically significant.

#### Effect on growth

Because reports suggested that *L. branchialis* affected growth of gadoids, changes in length, weight, and condition factor were utilised as a basis to compare growth between samples of control and infected cod. Among a group of young fish ( $\sim 33$  cm) examined at 4-wk intervals, total length, total body weight, and k-factor were significantly lower in fish harbouring 3 or more parasites from the fourth week onwards than among controls (Fig. 15a–c). These variables were also consistently lower but not always significantly different from controls in a group harbouring 1 or 2 parasites. Similarly, k-factor was also significantly different in 2 groups of infected subadult cod from controls that were sampled only 3 times following infection (Fig. 15d–f). Among the larger size classes of fish, k-factor, total length, and body weight were significantly lower in cod harbouring 3 or more parasites from 9 wk after infection and in the group of cod infected with 1 copepod after 15 wk, but this did not occur consistently in fish infected with 2 parasites (Fig. 16a–c). An exception to the above was noted among another group of adult cod that were infected with 1 copepod and held at water temperatures 2 C higher. Body weight and k-factor increased initially but decreased afterwards (Fig. 16d–f). The overall picture indicates, however, a trend toward a lower k-factor in parasitized cod with time especially in the small size classes and in the larger groups when 3 or more parasites were present (vide Fig. 17).

#### Effect on weight gained

Because it was observed that *L. branchialis* affected weight gained, studies were conducted to ascertain more precisely the effect of *L. branchialis* on weight gained in 2 groups (subadult and adult) of fish that were identified individually at intervals after infection. Small, parasitized fish ( $\sim 33$  cm) lost weight within the initial



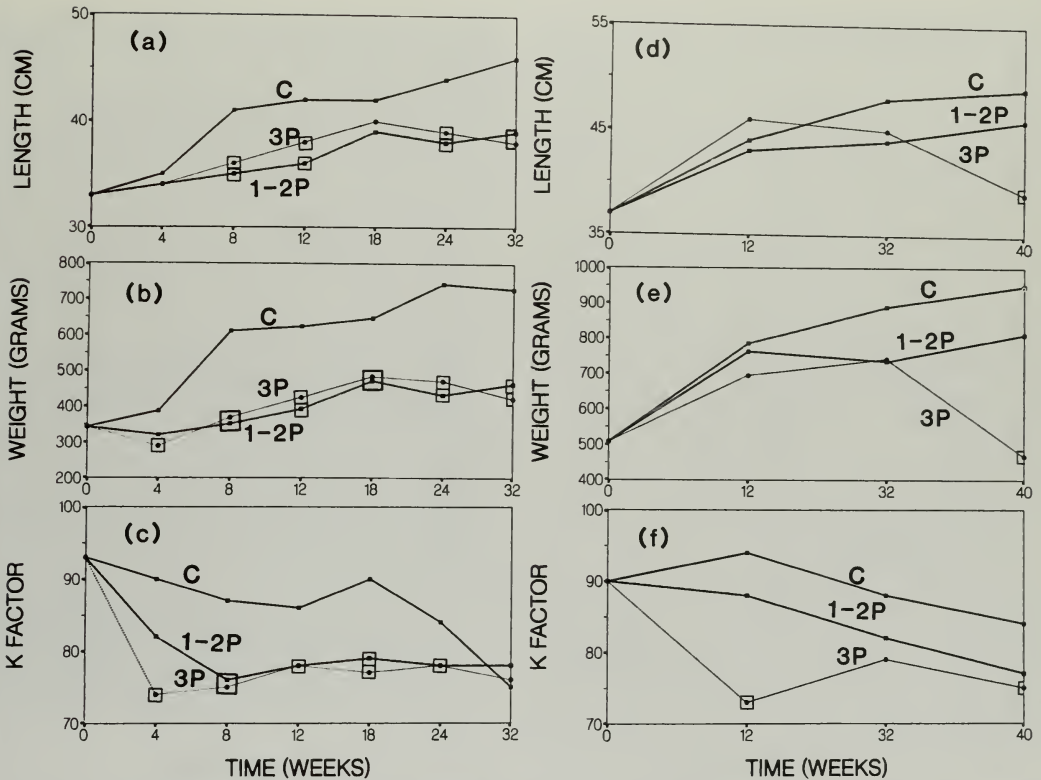


FIGURE 15. Effect of *L. branchialis* on mean body length, weight, and condition (k) factor of subadult Atlantic cod infected with 1-2 (1-2P) or 3 or more (3P) parasites. Some fish (a-c) were held in indoor tanks where the water temperature was 2 C higher than those held in a raceway (d-f). C = controls. In (a-c), n values are: C = 16, 1-2P = 15, 3P = 11, and (d-f), C = 16, 1-2P = 23, 3P = 11, respectively. Squares around mean values indicate significant differences ( $P < 0.05$ ) from controls.

4 wk, eventually gaining weight after this time but this was significantly lower than that of controls (Fig. 18a). In the larger size class (~56 cm), cod harbouring 1-2 parasites gained more weight initially than the controls, but subsequently this was lost (Fig. 18b). Fish infected with 3 parasites lost weight within the initial period, later gaining minimal weight that subsequently was lost. Among 2 additional groups of subadult and adult fish that were held in a raceway and sampled at intervals, parasitized fish, especially those infected with 3 or more parasites, weight gained was consistently lower than that of controls (Fig. 18c, d).

#### Effect on food consumed and weight gained

To ascertain the effect of parasitism by *L. branchialis* on food consumption and weight gain, 10 cod (32-40 cm) about 6 wk (mid-October, water temperature = 8 C, vide Fig. 1b) after infection were fed to satiation over a 28-day pe-

riod. Ten control cod, held separately, were treated similarly. Control cod consumed 45 g/feeding/host in contrast of 27 g/feeding among the infected group. Moreover, uninfected cod gained more weight (23%) than the infected fish (10%). This indicates that the conversion factor (weight gained/food consumed) in control fish was about twice (0.27) that of the infected group (0.14). A second feeding experiment was also conducted on another group of 10 cod of comparable size about 8 wk (early November, water temperature = 5 C) after infection for 28 days. Controls ate 44 g/feeding/host in contrast to 22 g/feeding/host consumed by the infected group. A weight gain of 28% was observed in the control group, whereas it was lower (9%) in the infected fish. The conversion factor was greater in the controls (0.33) than in the infected group (0.13). In a third trial involving 10 larger cod, infected ( $\bar{x}$  length,  $47 \pm 1$  cm) and uninfected ( $\bar{x}$  length,  $45 \pm 1$  cm) fish (10/group) were fed to satiation over a 28-

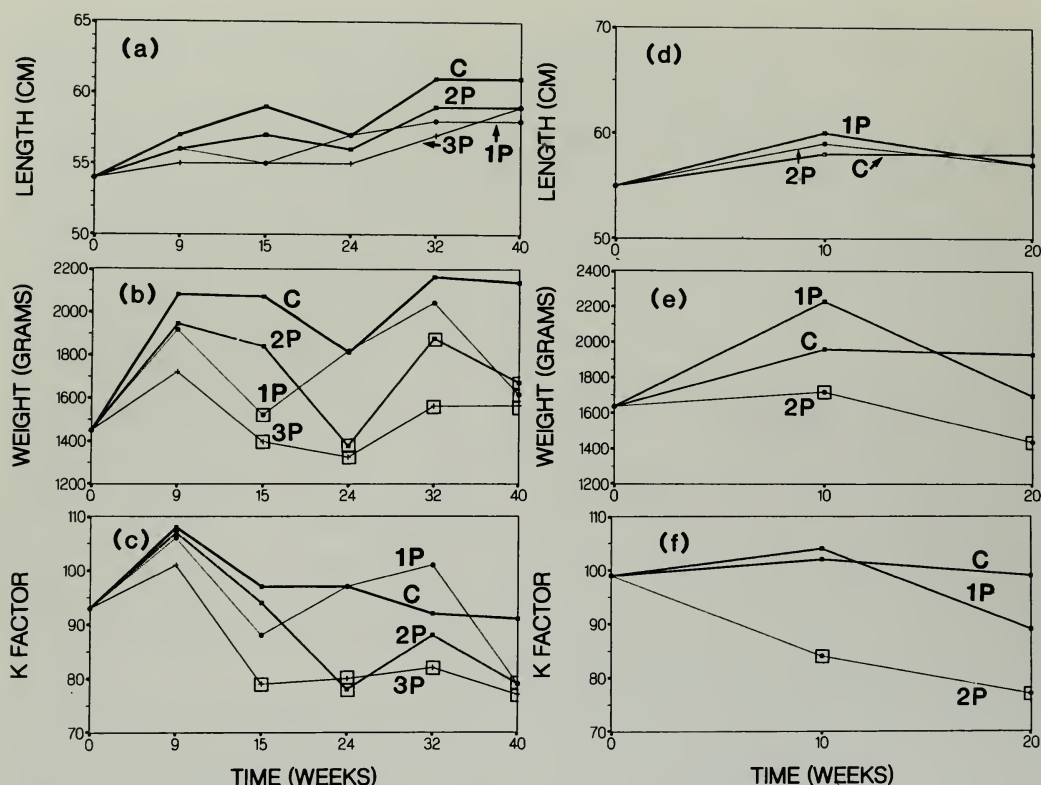


FIGURE 16. Effect of *L. branchialis* on mean body length, weight, and condition (k) factor of adult cod infected with 1 (1P), 2 (2P), 2 or more (>2P), or 3 or more (>3P) parasites. One group of fish (a-c) was held at the ambient water temperature in a concrete raceway, whereas the other (d-f) was kept in an indoor tank where the temperature was 2 C higher. C = controls. In (a-c), n values are C = 22, 1P = 14, 2P = 15, 3P = 17, and (d-f), C = 17, 1P = 11, 2P = 10. Squares surrounding mean values indicate significant differences ( $P < 0.05$ ) from controls.

day period (water temperature = 6 C). It was estimated that the mean food consumption was 63 and 54 g/feeding/host for the control and infected cod groups, respectively. When the experiment was terminated, weight gain differed between control (30%) and infected (22%) fish, and the conversion factor was greater in the control group (0.33) than in the infected (0.22) fish group.

#### Effect on reproduction

To ascertain the effect of *L. branchialis* on reproduction, control and infected cod were examined during the months of April and May for evidence of spermeation or ovulation. Among a group of 47 uninfected cod examined during April, 38% (18) were either spermeating or ovulating in contrast to 26% (9) of 34 infected fish. Only cod with single infections in the latter group were mature. Following examination 3 wk later

in May, the percentage of mature individuals increased in the control group to 63% (30) of the 48 examined, whereas it was substantially lower, 19% (9), in the infected group of 48 fish. Included among the infected males that were considered mature were 5 animals that were virtually spent. In contrast, all males in the control group were still spermeating and produced a copious amount of seminal fluid containing sperm.

A number of adult cod harbouring 2 or more parasites as well as uninfected controls were necropsied in the latter part of May to determine the effect of parasitism on gonadal somatic indices. The latter were significantly lower in both sexes of infected fish especially in female fish (Table VI).

#### Effect of stressors on *L. branchialis*-infected cod

To test the effect of stress on parasitized fish, groups of cod harbouring *L. branchialis* were ex-

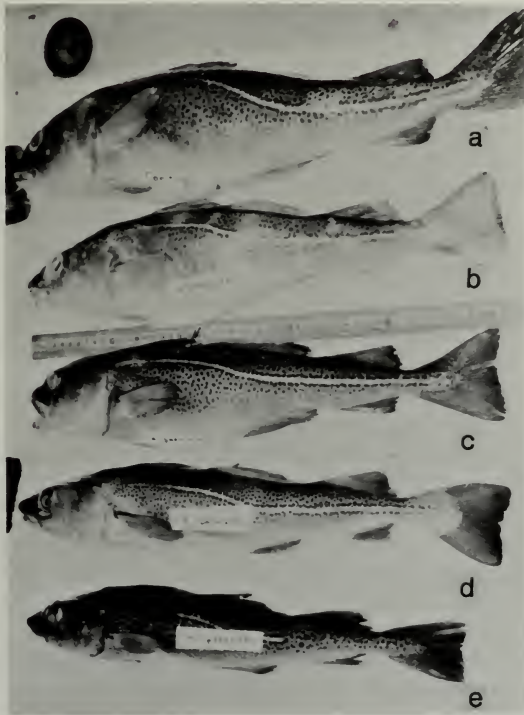


FIGURE 17. The effect of *L. branchialis* on growth of parasitized cod is shown in this photograph with a control (a), 9 mo after experimental infection. These fish were approximately the same length when the infection was initiated. Four of the fish harboured 1 (b), 2 (c), 3 (d), or 4 (e) parasites.

TABLE VI. Effect of *Lernaeocera branchialis* on gonadal somatic indices (GSI) of Atlantic cod 9 mo following experimental infection.

Fish group	n	GSI	
		Female	Male
Control	19	28.2 ± 2.5	15 18.0 ± 1.7
≥ 2 <i>L. branchialis</i>	18	9.2 ± 2.6*	12 13.2 ± 2.4*

\* Significantly different,  $P < 0.05$ , from controls.

posed to water-accommodated fractions of a crude oil or infected with a hemoprotozoan, *T. murmanensis*. Eight subadult cod, about 7 wk after infection with *L. branchialis*, were exposed to water-accommodated fractions of a crude oil at the level of about 100 µg/L. An uninfected group of comparable size was also exposed in the same oil tank (Table VII). An uninfected and an infected group were held under oil-free conditions. After 6 wk, the oil-treated fish were depurated for 6 wk. During this period, 3 of the 8 infected, oil-treated cod died. Surviving fish of the latter group lost weight (7.5%), whereas the 3 other groups of cod gained weight, especially those harbouring *L. branchialis* in which a 33% increment occurred.

A group of 11 adult cod, harbouring *L. branchialis* ( $1.6 \pm 0.2$ /fish) 8 mo after experimental infection, was also exposed to a higher concentration (~500 µg/L) of oil fractions. Three other

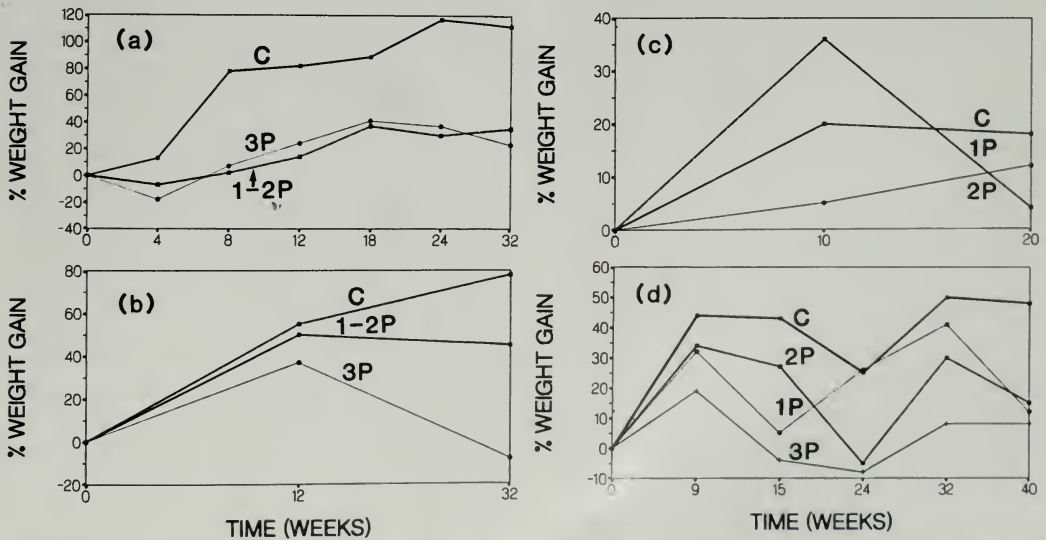


FIGURE 18. Comparison of mean percent weight gained in subadult (a, b) and adult cod (c, d) after infection with *L. branchialis*. Number of parasites (P) infesting each fish group is indicated by an Arabic numeral. C = controls. n values are the same as in Figures 15 and 16.



TABLE VII. *The effect of water-accommodated oil fractions (WAOF) and the hemoprotozoan, Trypanosoma murmanensis as stressors on Atlantic cod concurrently infected with L. branchialis.*

Stressor	Variable	Control	<i>L. branchialis</i>	WAOF (~100 ppb)	<i>L. branchialis</i> (~100 ppb) + WAOF*
WAOF (~100 ppb)	Length (cm)†	40 ± 1.4	35 ± 0.7	38 ± 1.0	38 ± 1.2
	Weight (g)	605 ± 37	322 ± 22	410 ± 64	439 ± 49
	% Weight change after 12 wk	5.3	33.0	15.6	-7.5
<i>T. murmanensis</i>	Variable	Control	<i>L. branchialis</i>	Trypanosomes	Trypanosomes + <i>L. branchialis</i> ‡
<i>T. murmanensis</i>	Length (cm)	43 ± 1.5	38 ± 0.9	43 ± 0.4	42 ± 0.7
	Weight (g)	713 ± 76	449 ± 32	742 ± 42	564 ± 14
	% Weight change after 12 wk	2.0	-1.0	-30	-14
WAOF (500 ppb)	Variable	Control	<i>L. branchialis</i>	WAOF	<i>L. branchialis</i> + WAOF
WAOF (500 ppb)	Length (cm)	54 ± 0.6	53 ± 0.9	51 ± 1.2	48 ± 1.7
	Weight (g)	1,333 ± 66	1,280 ± 71	1,148 ± 54	926 ± 47
	Condition (k) factor × 10 <sup>2</sup>	86 ± 3	86 ± 3	82 ± 4	70 ± 2§
	Liver s.i.    (× 10 <sup>2</sup> )	5.9 ± 0.5	4.5 ± 0.4#	6.8 ± 0.3	4.8#
	Heart s.i. (× 10 <sup>2</sup> )	1.6 ± 0.1	1.6 ± 0.1	1.9 ± 0.1	2.0 ± 0.2¶

\* Three died of 8 exposed/group.

† Initial length and weight.

‡ Nine died of 17 exposed/group.

§ Significantly lower ( $P < 0.05$ ) than all other groups.

|| s.i. = Somatic index.

# Significantly lower ( $P > 0.05$ ) than WAOF group.¶ Significantly greater ( $P > 0.05$ ) than control and *L. branchialis* groups.

corresponding control groups of fish of comparable size and/or infection with *L. branchialis* were held. Between 3 and 4 wk after exposure to oil, 10 of the infected fish died, whereas none occurred in the other 3 groups. At autopsy, the condition factor ( $70 \pm 2 \times 10^{-3}$ ) in these fish was significantly lower than that of the control ( $86 \pm 2 \times 10^{-3}$ ), *L. branchialis* ( $86 \pm 3 \times 10^{-3}$ ), or oil-treated ( $82 \pm 2 \times 10^{-3}$ ) groups. Additionally, the liver had decreased in size, whereas the heart was enlarged in the fish that succumbed (Table VII).

To ascertain the effect of *T. murmanensis* on subadult cod harbouring *L. branchialis*, a group of 17 cod, about 4 mo after infection with the copepod, were subsequently infected with the trypanosome via leeches. Corresponding control groups were held (Table VII). Within a 12-wk period, 9 fish infected concurrently with both parasites succumbed, whereas 4 in the trypanosome and 2 in the *L. branchialis*-infected groups died. No controls died and no weight loss was observed in this group. However, the 3 other groups decreased in weight, especially the trypanosome-infected fish, in which it was greatest (30%).

## DISCUSSION

Reconstruction of the life cycle of *L. branchialis* in the Newfoundland area is based on field and laboratory observations. Common lumpfish migrate inshore from the month of April onward to spawn and the males remain afterward for an additional 6–8 wk (July–September) to guard and aerate the egg mass (Leim and Scott, 1966). Peak intensities of the larvae of *L. branchialis* occur on lumpfish during July and August (Templeman et al., 1976). These larvae subsequently detach themselves, mature, and after a short free-swimming stage, during which copulation occurs, the females become attached to cod, probably in the latter part of August and through September. The population of cod, inshore at this time, is composed of young that have grown up in coastal areas and older, migratory fish. All are susceptible to *L. branchialis*. Examination of samples of parasites taken from cod, noted in a previous study (Khan and Lacey, 1986), revealed that development during autumn in the field appeared comparable to that noted in the present study (Figs. 2–13). Adult parasites, after releasing their eggs, then proceed to degenerate progressively in

the following 3–6 mo. Thus, the adult phase of the female's life cycle of *L. branchialis* is approximately 1 yr but the body of the parasite, after egg release, might remain attached to its host for periods up to 6 mo afterward. Several workers estimated this period accurately based on data obtained from field-infected fish (Stekhoven, 1936a, 1936b; Kabata, 1958; Templeman and Fleming, 1963), whereas other estimates varied from 3 mo to 3 yr (vide Kabata, 1979). Eggs subsequently released from these parasites hatch, and the larvae eventually infect lumpfish, which are territorial and sedentary at this stage, in spring of the following year.

The present study confirms suspicions of other authors (Scott, 1909; Debrosses, 1948; Kabata, 1958; Mann, 1970) that *L. branchialis* causes mortality in cod fishes and this occurs in cod at the level of 36%. Mortality was greatest among the smallest size classes (29–35 and 36–41 cm) than among larger fish. Most of the deaths occurred in the first 4 mo after infection and were associated with multiple infections. However, 23% of the fish that succumbed harboured single infections. Death in young fish was associated with emaciation, hemorrhage in the tissue adjacent to the "holdfast," open lesions, severe necrosis, and low hemoglobin values, whereas in adults it was caused probably as a result of occlusion of the ventral aorta and/or branchial arteries. Bilaterally opposite parasites, both located between the fourth and fifth branchial arches, were more likely to cause death by inducing severe hemorrhage and ultimately necrosis in the intervening region than other dual or bilateral infections. Sudden deaths 4 mo after infection were probably associated with thrombi and blockage of the major branchial blood vessels.

Fish that survived infections with *L. branchialis* also showed reduced weight gain and growth. This was apparent in small fish from the fourth week following infection, but it also occurred at later intervals (~10 wk) in larger size groups with multiple infestation. Infected fish consumed less food than control fish, had lower weight increments, and the conversion factor was also lower in this group. Infected fish became anemic and had lower total plasma proteins than the uninfected groups. Liver somatic indices decreased, especially in small fish, but the spleen and heart were apparently unaffected. It can, therefore, be assumed that metabolic demands of the parasite might have been responsible for

this difference as suspected by Kabata (1958, 1979, 1984).

Abnormal behaviour was demonstrated by cod infected with *L. branchialis*. Infected cod were hyperactive and swam in an erratic manner. Some remained at the surface of the water because of an excess of air that was present in the swim bladder. There are reports that the low prevalence of *L. branchialis* in offshore areas might be attributed to the fact that infected fish remain close to shore (Sproston and Hartley, 1941; Kabata, 1958). Sproston and Hartley (1941) have speculated that the parasites induce abnormal intake of fluid caused by metabolic alterations and consequently remain in a low salinity estuary. Possibly, electrolyte levels were low in infected fish and conceivably could have been the underlying cause for the fish remaining in the low salinity areas. In the Newfoundland area cod rarely frequent estuaries. The low prevalence offshore (noted by Templeman et al., 1976) might, therefore, be the result of (1) mortality, caused by the parasite, (2) an inability to swim deep caused by retention of excessive air in the swim bladder, (3) predation of debilitated fish during migration as infected fish utilise less oxygen than uninfected fish (Mann, 1953), and/or (4) a change in blood sugar and electrolyte levels both of which could affect mobility.

A reduction in the intensity of *L. branchialis*, which varied from 1.2 to 1.7 per host, was observed in both juvenile and adult cod in the present study. This reduction, which occurred in 17–27% of the fish sampled, was more pronounced when multiple infections were present. However, only a small percentage (4) of the cod eventually became parasite-free. In contrast, Shields and Goode (1978) reported rejection of viable *Lernaea cyprinacea* from its host, *Carassius auratus*, prior to egg production. Parasites were eliminated from 100% of the goldfish and up to 90% of the copepods were shed within 11 days after attachment. Also, about 33% of 18 fish became parasite-free. Histological studies revealed that a massive inflammatory response against the invasive parasites was associated with parasite rejection and possibly was responsible for immunity against reinfection. The latter, however, did not occur in cod as 63% became reinfected with *L. branchialis* following exposure.

There are conflicting opinions about the pathogenicity of *L. branchialis* in gadoids. Pathological changes were observed in some infected



hosts (Stekhoven, 1935, 1936b; Stekhoven and Punt, 1937; Mann, 1953; Kabata, 1958; Sundnes, 1970; Van den Broek, 1978), whereas only minimal changes were found in others (Sproston and Hartley, 1941; Sherman and Wise, 1961; Khan and Lacey, 1986). These differing opinions can be attributed to the time of the year at which the sampling was conducted. Major changes, especially on condition factor, would be readily detected in infected young fish at any time of the year (October through June). However, in older groups infected with 1 parasite and examined in summer (some 10 mo after infection), this might not be readily apparent as these fish will have had sufficient time to adjust to the infection. Additionally, *L. branchialis* infecting hosts in European waters favours attachment to the bulbus arteriosus, whereas in the northwestern Atlantic, the branchial arteries and ventral aorta seem to be the preferred sites. Despite these conflicting opinions, an infected fish, even with 1 parasite, can succumb readily although blood and other values appear normal. Stress appears to be the main factor that could affect infected fish. Mann (1953) has shown that infected fish use less oxygen and it could be assumed that these fish would die more readily than uninfected fish when the supply of oxygen is limited. Cod captured in cod traps located in a cove about 20 km from our laboratory have been transported to holding tanks in an aerated tank-truck during summer of each year. In most years, most of the *L. branchialis* infected cod (~30–60 cm) died within 24 hr or after arrival. Whenever oxygen levels fell below the normal or there were sudden changes in water temperature (~4 C) caused by upwelling of cold water in summer, deaths among the infected fish consistently preceded those of uninfected group. During a period of supersaturation more recently (April 1987), 48 infected (1–4 parasites/fish) cod died about 36 hr prior to the first uninfected fish before the cause of death was detected. Similarly, about 80% mortality (9) was noted among a group (11) of infected cod (47–58 cm) that were exposed to water-accommodated oil fractions (~500 µg/L) within 4 wk whereas no uninfected fish died. These infected fish, each harbouring 1 or 2 parasites, were exposed to oil fractions in the summer, i.e., approximately 8 mo after infection. Moreover, subadult cod harbouring parasites also succumbed when exposed to lower hydrocarbon concentrations. This increased susceptibility to additional stress was evident when similar sub-

adults, harbouring *L. branchialis*, were infected with another parasite, *T. murmanensis*. About 60% of the fish succumbed and the surviving fish were emaciated to the extent that survival in nature would be inconceivable. It appears, then, that fish infected with *L. branchialis* are more likely to die when stressed, a suspicion raised by some authors previously (Mann, 1970; Kabata, 1984; Khan and Lacey, 1986).

The present study also provides evidence that *L. branchialis* delays gonadal maturation in cod with multiple infections. Kabata (1958) observed that infected female haddock had smaller gonadal weights and Hislop and Shanks (1979) noted that fecundity in infected haddock was 21% lower than in uninfected fish. Templeman et al. (1976) also reported that *L. branchialis* interfered with the onset of sexual maturity of cod. Probably some aspect of the reproductive cycle is affected, delaying temporarily or disrupting maturation. Because the spawning period of cod, in the northwestern Atlantic, is of short duration (1–2 wk), any delay in gonadal maturation is likely to prevent spawning or spermeation and ultimately affect recruitment.

In the present study, 18% (12) of 68 cod infected with 1 *L. branchialis* died after infection and deaths were higher (40%) in young fish (~34 cm). Moreover, body condition of the fish that survived was low (vide Table VI) and many were emaciated. During summer, when transmission of the parasite occurs, a greater part of the population of cod in coastal areas is composed of young fish. Templeman et al. (1976) noted that 86% of a sample of 322 fish (<40 cm) harboured 1 parasite and it is assumed, from the present study, that these fish were infected in the previous year. Moreover, in an area on the southwest coast of Newfoundland, it was estimated that about 20% of the sampled population harboured *L. branchialis* (cf. Templeman et al., 1976; Khan and Lacey, 1986). Mann (1953) reported that 80% of the fish <35 cm were infected with *L. branchialis*, whereas in a Norwegian fiord, prevalence was approximately 27% (Sundnes, 1970). Sundnes (1970) estimated at least 25% mortality in cod 4–5 yr old and an overall 6% mortality higher than the natural mortality in uninfected fish of comparable age. Based on deaths from our study, it appears, then, that there are potentially substantial losses through mortality from infections with *L. branchialis* as well as weight loss through morbidity in surviving



fish. Studies are currently in progress to relate our laboratory results to those observed in the field in order to ascertain the impact of *L. branchialis* on cod stocks.

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## COMMERCIAL BLAST-FREEZING OF THIRD-STAGE *ANISAKIS SIMPLEX* LARVAE ENCAPSULATED IN SALMON AND ROCKFISH

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**ABSTRACT:** Sixty-four fish were blast-frozen to  $-35^{\circ}\text{C}$  for 15 hr to determine the effects of commercial blast-freezing on the viability of third-stage larvae of *Anisakis simplex* encapsulated in the muscle and viscera of sockeye salmon (*Oncorhynchus nerka*) and canary rockfish (*Sebastes pinniger*). Parallel tests were conducted on larval nematodes in 16 whole (round) salmon, 16 dressed salmon (heads and viscera removed), and 32 whole (round) rockfish. After blast-freezing, 4 in-the-round salmon, 4 dressed salmon, and 8 in-the-round rockfish were examined at 1, 24, 48, and 72 hr. A total of 3,539 dead and 6 live larvae were collected from the fish tissues after standard enzymatic digestion. Salmon were infected with 1,245 of these larvae, and rockfish with 2,300. The 6 live worms, 2 from salmon and 4 from rockfish rounds, were recovered from muscle 1 hr after freezing; they were slightly motile and showed severe internal damage. No viable worms were found at or after 24 hr. The commercial blast-freezing process effectively killed larval nematodes in whole or dressed fish. Market-ready samples of previously blast-frozen silver salmon (*O. kisutch*) and chum salmon (*O. keta*) fillets and chum salmon steaks yielded no live worms, thereby confirming the efficacy of this process.

The ingestion of live third-stage larvae of *Anisakis simplex*, *A. physeteris*, *Pseudoterranova decipiens* (= *Phocanema d.*), and other anisakid nematodes may cause anisakiasis in humans. Much attention has been given to documenting the occurrence, treatment, and epidemiology of this zoonotic disease (Yokogawa and Yoshimura, 1967; Oshima, 1972, 1987; Margolis, 1977; Hafsteinsson and Rizvi, 1987); however, few studies have addressed preventive measures to ensure that fishes or other seafood products eaten raw or partially cooked are safe.

Freezing the intermediate host for specified lengths of time has been effective in killing the third-stage larvae of *A. simplex* encapsulated in marine fishes (Gustafson, 1953; Bier, 1976; Hauck, 1977; Deardorff et al., 1984). Most of these studies used domestic freezers to determine the resistance of the larvae to various temperatures.

Although domestic freezers are used by the general public, they do not simulate the methods generally used by the commercial fishing industry. Blast-freezing, a process that rapidly freezes the fishes to  $-40^{\circ}\text{C}$ , is now a common practice for much of the salmon industry. The temperature during the blast-freezing process decreases

rapidly; it is significantly colder than that of typical domestic freezers and therefore may be more effective in killing the third-stage larvae present in infected fishes.

In the present study, fresh fish were blast-frozen at a commercial facility and examined at specific time intervals to determine whether the process represents a means of decreasing the possibility of infection. Salmon and rockfish were chosen for testing because these fish are most often implicated in the United States in the transmission of infective *Anisakis* larvae to humans (Valdiserri, 1981; Deardorff et al., 1986, 1987), and they have a high prevalence of larval anisakines (Myers, 1979; Dailey et al., 1981; Jensen et al., 1982).

### MATERIALS AND METHODS

A total of 64 fish, 32 sockeye salmon (*Oncorhynchus nerka*) and 32 canary rockfish (*Sebastes pinniger*), caught by commercial fishermen in the Puget Sound or offshore of Bellingham, Washington, in September 1986, were examined. The salmon weighed 2.7-3.6 kg; the rockfish weighed 1.8-2.7 kg. (One rockfish weighed 9.0 kg.) Of the 32 whole salmon, 16 were dressed (heads and viscera removed) before freezing; the remaining 16 salmon and all rockfish were frozen in the round (whole fish).

Blast-freezing was conducted at The Pacific Salmon Company, Inc. (Seattle, Washington). Our protocol closely simulated their usual handling and processing procedures. Briefly, round and dressed fish were placed in single layers on aluminum racks and frozen for 15 hr. The blast-freezer was set at  $-40^{\circ}\text{C}$ ; however, the actual temperature attained during the process was  $-35^{\circ}\text{C}$ .

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TABLE I. The effect of commercial blast-freezing on the viability of the third-stage larvae of *Anisakis simplex* infecting sockeye salmon and canary rockfish at various time intervals.

Sample	No. fish examined per time period	Time examined after blast-freezing				Totals
		1 hr	24 hr	48 hr	72 hr	
Salmon rounds	4					
Viscera		32* (0)†	44 (0)	52 (0)	52 (0)	180 (0)
Muscle		92 (2)	111 (0)	199 (0)	152 (0)	554 (2)
Salmon dressed	4	68 (0)	198 (0)	203 (0)	40 (0)	509 (0)
Salmon totals	8	192 (2)	353 (0)	454 (0)	244 (0)	1,243 (2)
Rockfish rounds	8					
Viscera		1,252 (0)	92 (0)	132 (0)	142 (0)	1,618 (0)
Muscle		227 (4)	88 (0)	122 (0)	241 (0)	678 (4)
Rockfish totals	8	1,479 (4)	180 (0)	254 (0)	383 (0)	2,296 (4)

\* Total number of dead larvae recovered.

† Value in parentheses represents total number of viable larvae.

C, as determined by the process control thermometer. Fish were removed from the freezer, individually glazed with a sugar and water solution to "seal" the fish and thus prevent oxidation and dehydration of the muscle tissue, and stored at  $-18^{\circ}\text{C}$ . Four round and 4 dressed salmon and 8 round rockfish were removed from the freezer at 1, 24, 48, and 72 hr after freezing; they were allowed to thaw in cold water, filleted, and digested. For controls, 2 round salmon and rockfish were examined at each time interval. All control fishes were infected; all recovered worms were viable.

Additionally, 16 salmon strips, either silver salmon (*O. kisutch*) or chum salmon (*O. keta*), and 16 steaks (all *O. keta*) that had been blast-frozen and prepared for sale at commercial supermarkets were purchased from The Pacific Salmon Co. These market-ready products had undergone blast-freezing within the previous 7 days. The steaks, in standard vacuum-packs and strips (fillets), were shipped frozen from Seattle to Dauphin Island, Alabama, and were immediately thawed and examined.

The digestion methodology is modified from the Food and Drug Administration's *Bacteriological Analytical Manual* (1984). Fish tissues were placed into 3- or 4-L beakers containing a magnetic stirring bar and approximately 2 L of digest solution. The solution consisted of 40 g of pepsin per liter of water, adjusted to pH 2 with HCl. For each fish, all the musculature and viscera, when present, were digested separately. Tissues were in the digest solution from 4 to 6 hr. After digestion, the digested remains were poured into a pan and examined for larval nematodes. Market-ready steaks and strips were individually digested as previously described.

All larvae removed from the remains of the digest were placed in phosphate-buffered saline solution at ambient temperature, counted, and assessed for viability. A larva that showed movement after being stimulated with a dissection needle was considered to be alive. The presence or absence of movement in all helminths was confirmed 2–4 hr later under a dissecting microscope. For identification, representative larvae were fixed in glacial acetic acid, stored in 70% ethyl alcohol, cleared in lactic acid, and examined under a compound microscope.

## RESULTS

The effects of blast-freezing on the viability of the third-stage larvae of *A. simplex* are presented in Table I. All fishes were infected with third-stage larvae of *A. simplex*. Of the 3,545 larvae digested from the salmon and rockfish tissues, only 6 worms (2 from salmon and 4 from in-the-round rockfish) showed signs of coiling and uncoiling. These worms were recovered from the musculature at 1 hr. Without tactile stimulation, however, these nematodes showed no movement; their internal structures were severely damaged. Tissue disruption, including numerous areas of dissociation of esophageal and intestinal tracts, was observed. No further movement of these or other recovered worms was detected.

The 32 round or dressed salmon were infected with 1,245 worms; 734 worms (i.e., 46 worms per fish) were found in the round salmon. Of these, 75% of the larvae were in the muscle and 25% in the viscera. The 16 dressed salmon yielded 509 larval nematodes, or about 32 worms per fish.

The 32 rockfish were infected with 2,300 *Anisakis* larvae, or 72 worms per fish; 75% were in

TABLE II. Percentage, average, and range of larval *Anisakis simplex* in market-ready salmon.

Item	% Infected	Average sample weight (g)	Average worms/sample (range)
Steaks* (n = 16)	81	258	4 (0–14)
Strips† (n = 16)	38	393	1 (0–4)

\* From *O. keta*.† From *O. kisutch* and *O. keta*.



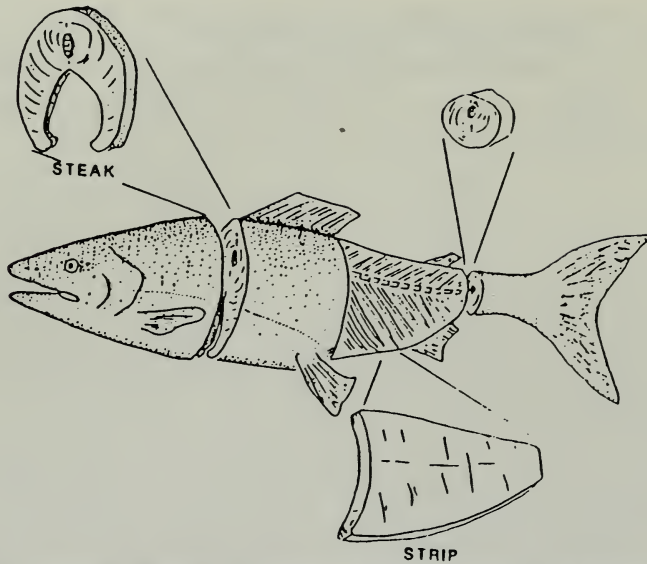


FIGURE 1. Diagram of salmon, showing location and shape of standard steak and strip. Circular steak less common.

the viscera and 25% in the musculature. Four viable worms and 1 dead third-stage larva of *P. decipiens* were collected from the musculature of the 9-kg rockfish at 1 and 24 hr, respectively. Findings on the viability of larval *A. simplex* in commercially prepared fillets and steaks are presented in Table II. All recovered worms were dead.

#### DISCUSSION

Commercial blast-freezing was demonstrated to be extremely effective in rendering larval nematodes inactive. More than 99% of the recovered *Anisakis* larvae infecting *O. nerka* and *S. pinniger* were dead at 1 hr after completion of the blast-freezing process. Also, the finding of a dead third-stage larva of *P. decipiens* at 24 hr suggests that blast-freezing may also be effective in killing that invasive larval type.

The cold tolerance of the 6 surviving helminths may be attributed to thickness of the fish. Surviving worms were found only in the muscle of fish, with 4 of the 6 worms from the musculature of the largest rockfish round (9 kg). These data suggest that because no live worms were recovered from dressed fishes, the presence of viscera may slow down the freezing process in fish in the round. The higher lipid content of salmon, compared with that of rockfish, apparently does not affect larval survival.

No matter how these 6 worms avoided death,

the deleterious effects of blast-freezing were evident. No worms were able to move voluntarily; external stimulation was necessary for slight muscular contraction. Further, the severe damage to the internal morphological structures of these worms may have been caused by the fast cooling and subsequent formation of intracellular ice. We believe, therefore, that these 6 slightly mobile larvae would not be able to penetrate tissue.

Compared with strips, salmon steaks expose the consumer to more worms because of the higher percentage of infection in a smaller serving portion. Steaks are usually cut from the region that includes the "belly-flap" of the salmon (Fig. 1). The area of musculature nearest that abdominal cavity is suspected of containing more worms. Steaks taken posterior to the abdominal area are circular and, if this hypothesis is valid, would have fewer encapsulated worms. In our study, fillets had fewer worms, probably because most of the musculature in a fillet is not adjacent to the abdominal cavity and/or the filleting process generally leaves the rib bones and associated musculature of the abdominal cavity on the carcass of the fish. Our study supports the hypothesis that more worms are found in the abdominal musculature than in the posterior region.

Although this is the first study to test commercial blast-freezing as a method to kill larval *Anisakis*, Gustafson (1953) did test the effec-

tiveness of a commercial freezer. After freezing herring in 100-lb blocks to about  $-30^{\circ}\text{C}$  for 16 hr, he stored the blocks at  $-12^{\circ}\text{C}$  until used. At 24 hr, he found 41 of the 361 (9%) worms alive; no worms were found to be alive at subsequent time intervals. It is difficult to compare or contrast the present experiments with those of Gustafson because of several variables, such as differences in species and sizes of fishes, protocols (blocks of ice vs. individual freezing), and temperatures. Nevertheless, both studies found that the extremely cold temperatures attained by using commercial equipment for a relatively short time had a lethal effect on third-stage *Anisakis* larvae.

Deardorff (1986) regards freezing seafood products as the most promising preventive measure against infection with anisakid larvae. This research supports that statement and underscores the many benefits of blast-freezing. Not only does blast-freezing kill parasites, it is cost-effective, causes little change in the flavor or texture of the fishes, and is of practical importance because the commercial fishing industry currently uses the process.

Market-ready samples of fish that had been blast-frozen yielded no live worms, indicating that routine commercial handling and freezing of these products increased the margin of safety to the consumer, regardless of how the fish would be prepared for consumption. The evidence presented here indicates that blast-freezing to at least  $-35^{\circ}\text{C}$  for 15 hr could effectively prevent anisakiasis when thorough cooking of seafoods is not desired.

#### ACKNOWLEDGMENTS

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# SINGLE AND CONCURRENT INFECTIONS OF THE GOLDEN HAMSTER, *MESOCRICETUS AURATUS*, WITH *ECHINOSTOMA REVOLUTUM* AND *E. LIEI* (TREMATODA: DIGENEA)

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**ABSTRACT:** Single or concurrent infections of the intestinal trematodes *Echinostoma revolutum* and *E. liei* were studied in the golden hamster (*Mesocricetus auratus*). In single infections, some hamsters were fed  $25 \pm 5$  metacercarial cysts and others  $100 \pm 25$  cysts of either *E. revolutum* or *E. liei*. In concurrent infections, hamsters were fed simultaneously  $20 \pm 5$  metacercarial cysts of *E. revolutum* and  $20 \pm 5$  cysts of *E. liei* or  $100 \pm 25$  cysts each of both trematodes. All hamsters exposed singly to *E. revolutum* or *E. liei* were infected. In concurrent infections, 9 of 10 hamsters were infected with both species of echinostomes, and the ratio of *E. revolutum* to *E. liei* was 3:1. In single infections, 80% of the *E. liei* and 60% of the *E. revolutum* were in the posterior third of the small intestine. In concurrent infections, 80% of the *E. liei* were in the posterior third and 57% of the *E. revolutum* in the middle third of the small intestine. The histopathological response of *E. liei* and *E. revolutum* in single and concurrent infections showed erosion of intestinal villi with lymphocytic infiltration as the primary response. Extraintestinal echinostomiasis occurred in 2 of the infection groups. Differences in hemoglobin and packed cell volume occurred in the different infection groups.

Infectivity, growth, and development of *Echinostoma revolutum* and *E. liei* in the golden hamster have been studied by Franco et al. (1986) and by Jeyarasasinghan et al. (1972), respectively. Clinical and pathological effects of *E. revolutum* in the golden hamster have also been reported (Huffman et al., 1986). Relatively few experimental concurrent infection studies on adult trematodes are available. Barus et al. (1974) reported on the interactions between *E. revolutum* and *Echinoparyphium recurvatum* in the hamster, and Zajicek (1968) found that susceptibility of birds to metacercaria of *Cotylurus cornutus* was influenced by infection with other trematode species. Fried and Gainsburg (1980) reported on the concurrent infection of the cecal trematodes *Zygocotyle lunata* and *Notocotylus* sp. in the domestic chick. Nollen et al. (1975) studied the inseminatory behavior of *Philophthalmus megalurus* and *P. hegeneri*, and *P. megalurus* and *P. gralli* (Nollen, 1984) in concurrent infections in chicks. In the present study, worm location, number of worms recovered, and the pathological effects of both single and concurrent infections of *E. liei* and *E. revolutum* are reported.

## MATERIALS AND METHODS

Laboratory-infected snails were the source of encysted metacercariae: *E. revolutum* from the kidney of *Physa heterostrophia*, and *E. liei* from the kidney and pericardium of *Biomphalaria glabrata*. Twelve-month-old golden hamsters (*Mesocricetus auratus*) were used and were provided food and water *ad libitum*.

In concurrent infections,  $20 \pm 5$  cysts of *E. revolutum* and  $20 \pm 5$  cysts of *E. liei* in distilled water were fed per os simultaneously to each of 10 hamsters. In single infections,  $25 \pm 5$  cysts of *E. revolutum* were fed to each of 10 hamsters and  $25 \pm 5$  cysts of *E. liei* were fed to each of 10 hamsters. Two animals from each group were necropsied on days 7, 11, 14, 18, and 21 postinfection (PI). The intestine was divided into 3 segments, duodenum, jejunum, and ileum (I, II, and III, respectively), and worm recovery per segment was noted.

To assess the pathological effects of heavy concurrent versus heavy single infections of *E. liei* and *E. revolutum*, 7 hamsters were infected with  $100 \pm 25$  cysts of *E. revolutum*, 7 hamsters were infected with  $100 \pm 25$  cysts of *E. liei*, and 6 with  $100 \pm 25$  cysts of both *E. liei* and *E. revolutum*. Four hamsters were designated as controls and not fed cysts. Hamsters were weighed and blood samples taken via cardiac puncture prior to exposure and on days 6 and 13 PI. A 3-ml syringe with a 25-gauge needle (Desai, 1968), rinsed with heparin, was used to collect the blood that was transferred to heparin-coated 3-ml Vacutainers (American Scientific Products, Edison, New Jersey) and refrigerated until processed. Packed cell volume (PCV) and hemoglobin concentration were determined for each sample on the day of collection. Air-dried blood smears were fixed and stained using Harleco Diff Quick (American Scientific Products) system and examined microscopically. Hamsters were examined daily for changes in behavior and physical appearance. Fecal samples were examined for the presence of diarrhea and parasite eggs. The hamsters were necropsied on day 13 PI.

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FIGURE 1. Live *Echinostoma revolutum* (left) and *E. liei* 14-day-old adults from a concurrent infection. Scale bar = 1 mm.

TABLE I. Worm recovery and distribution of single infections of *Echinostoma liei* and *E. revolutum* in the golden hamster.

Days postinfection*	No. of worms in intestinal segments: duodenum (I), jejunum (II), and ileum (III)				
	I	II	III	Totals	% Recovery
<i>Echinostoma liei</i>					
7	0	9	33	42	84
11	0	7	24	31	62
14	0	0	26	26	52
18	0	0	16	16	32
21	0	0	21	21	42
Total	0	16	120	136	54.4
% Recovery/segment	0	11.8	88.2		
<i>Echinostoma revolutum</i>					
7	0	0	7	7	14
11	0	8	30	38	76
14	4	6	0	10	20
18	0	7	12	19	38
21	0	6	8	14	28
Total	4	27	57	88	35.2
% Recovery/segment	4.5	30.7	64.8		

\* Two hamsters were used at each day postinfection and each animal was exposed to  $25 \pm 5$  metacercariae.

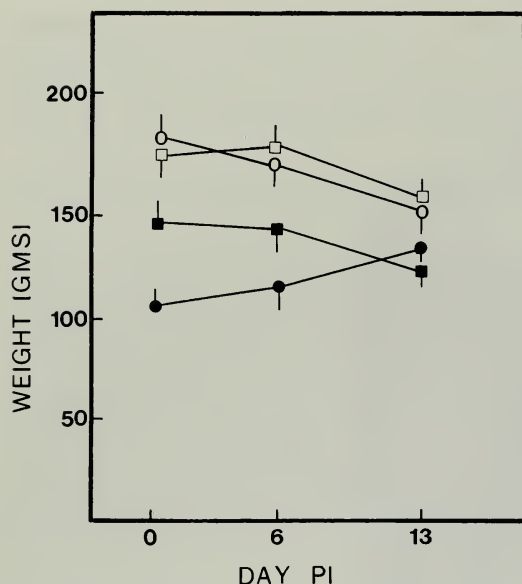


FIGURE 2. Weight loss in golden hamsters infected concurrently with  $100 \pm 25$  cysts of *Echinostoma revolutum* and  $100 \pm 25$  cysts of *E. liei* (○), singly with  $100 \pm 25$  cysts of *E. liei* (□), singly with  $100 \pm 25$  cysts of *E. revolutum* (■), and controls (●).

Gross lesions were recorded. The digestive tract was examined for echinostomes, and the number of parasites was recorded. Livers, gall bladders, spleens, and intestinal tissues were fixed in 10% natural buffered formalin (NBF), dehydrated in an alcohol series, embedded in paraffin, sectioned at  $6 \mu\text{m}$ , and stained with hematoxylin and eosin.

## RESULTS

In concurrent infections, living specimens were easily identified by the larger body, more distinct testes, and less attenuated posterior region in *E. revolutum* than in *E. liei* (Fig. 1).

Results of single infection with *E. liei* are summarized in Table I. One hundred percent of the hamsters were infected, and the number of par-

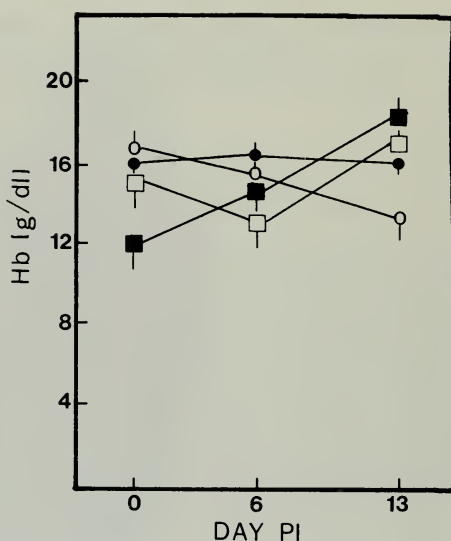


FIGURE 3. Hemoglobin values (g/dl) in golden hamsters infected concurrently with  $100 \pm 25$  cysts of *Echinostoma revolutum* and  $100 \pm 25$  cysts of *E. liei* (○), singly with  $100 \pm 25$  cysts of *E. liei* (□), singly with  $100 \pm 25$  cysts of *E. revolutum* (■), and controls (●).

asites recovered ranged from 2 to 28 (avg. 13.6). The worms were clustered mainly in the posterior third of the small intestine.

The single infection results with *E. revolutum* are summarized in Table I. All the hamsters were infected and the range of parasites was 2–23 (avg. 8.8). The worms were found singly, in pairs, and in clusters mainly in the posterior and middle third of the small intestine.

Table II summarizes the results of concurrent infections. Nine of the 10 concurrently exposed hamsters were infected with both species. One hamster was infected with only *E. revolutum*. The total number of worms recovered ranged from 7 to 27 (avg. 13.9) with a ratio of 3 *E.*

TABLE II. Worm recovery from concurrent infections with *Echinostoma revolutum* and *E. liei* in the golden hamster.

Days postinfection*	No. of worms in intestinal segments: duodenum (I), jejunum (II), and ileum (III) <i>E. revolutum</i> / <i>E. liei</i>			Totals	% Recovery	Ratio of <i>E. revolutum</i> / <i>E. liei</i>
	I	II	III			
7	0/0	6/1	7/4	13/5	32.5/12.5	2.6
11	1/0	8/0	7/6	16/6	40/15	2.6
14	0/0	17/6	14/3	31/9	77.5/22.5	3.4
18	0/0	6/0	8/5	20/5	50/12.5	4.0
21	1/0	16/0	6/10	23/10	57.5/25	2.3
Total	2/0	59/7	42/28	103/35	51.5/17.5	2.9

\* Two hamsters were used at each day postinfection. Each animal was exposed to 20 *E. revolutum* and 20 *E. liei* simultaneously.



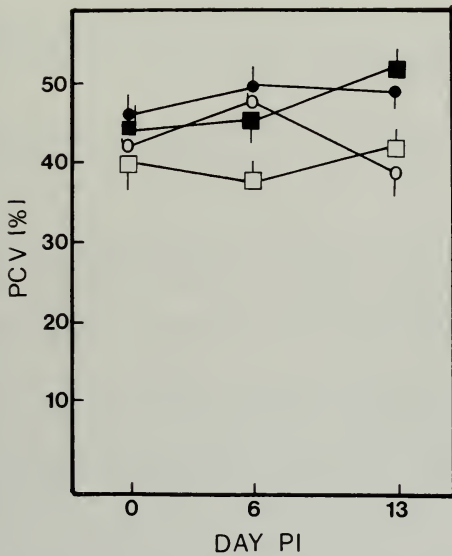


FIGURE 4. Packed cell volume (PCV) (%) in golden hamsters infected concurrently with  $100 \pm 25$  cysts of *Echinostoma revolutum* and  $100 \pm 25$  cysts of *E. liei* (□), singly with  $100 \pm 25$  cysts of *E. liei* (○), singly with  $100 \pm 25$  of *E. revolutum* (■), and controls (●).

*revolutum* to 1 *E. liei* in concurrently infected hamsters.

In single infections more than 60% of all worms from both species were in the posterior third of the intestine. In *E. liei* infections 88% of the worms recovered were in the posterior third and the other 12% in the middle segment. In *E. revolutum* infections 65% of the worms were in the posterior third, 30% in the middle segment, and 5% in the anterior third.

In concurrent infections, worms from both species were in the middle and posterior third of the small intestine. In the anterior third, 0% were *E. liei* and 1.9% were *E. revolutum*. In the middle third, 57% were *E. revolutum* compared to 20% *E. liei*. In the posterior third, 41% were *E. revolutum* compared to 80% *E. liei*.

In heavy concurrent and single infections, i.e., hosts with more than 35 echinostomes, hamsters developed progressive unthriftiness, unkempt hair coat, weakness, unsteady gait, and watery and bloody diarrhea. Parasite eggs were found in stools collected on day 11 in all infected hamsters. The clinical signs exhibited by hamsters infected with less than 35 worms were not as severe as those with greater than 35 worms.

The histopathological response of *E. liei* and *E. revolutum* in single and concurrent infections

showed erosion of intestinal villi with lymphocytic infiltration as the primary response.

Weight loss occurred in the 3 heavy dose experimental groups (Fig. 2). In the heavy dose concurrent infections suppurative lesions were observed on 50% of the livers, and *E. revolutum* was also found in the gall bladders of 50% of the infected hamsters.

Suppurative lesions and the presence of *E. revolutum* in the gall bladder of single infections also occurred. No liver lesions or the presence of *E. liei* was noted in the heavy dose single infections.

Hamsters infected with *E. revolutum* showed increases in hemoglobin (Hb) concentration (g/dl) and packed cell volume (%) (Figs. 3, 4). Peripheral blood smears from all infected animals were normal; no increase in eosinophils was noted. In single *E. liei* infections Hb (g/dl) and PCV (%) values decreased (Figs. 3, 4). Concurrent infections showed increases in both Hb (g/dl) and PCV (%) values.

## DISCUSSION

In the present study the distribution of *E. liei* in the small intestine was about the same in single or concurrent infections. Concurrent infections with *E. revolutum* differed from single ones in that there was a tendency to move from segment III (ileum) to segment II (jejunum). Reasons for this apparent anterior migration are not known; perhaps *E. revolutum* is migrating away from the densest population of *E. liei*.

The number of *E. liei* recovered in concurrent infections was markedly reduced, an observation in accord with the reduced recovery of *Notoctylus* sp. in the presence of *Zygocotyle lunata* (Fried and Gainsburg, 1980). Holmes (1961, 1962) found that worm distribution in the host gut was affected by concurrent infections with *Moniliformis dubius* and *Hymenolepis diminuta*. Excretory-secretory products from *E. revolutum* may adversely affect the survival of *E. liei* in concurrent infections in hamsters. In concurrent infections, physiological and/or space limitations may be imposed on the parasites.

The hamsters lost weight in the heavy dose experimental groups. This was due to severe diarrhea and consequent fluid loss in these hamsters.

Both parasites denude the epithelial villi of the small intestine. *Echinostoma liei* pathology differs from that of *E. revolutum* in that hemorrhage

occurs in *E. liei* infections. This hemorrhage is seen in association with the damage to the intestinal villi. These 2 trematodes also differ in that *E. liei* does not migrate into the common bile duct and does not infect the liver or gall bladder as does *E. revolutum*.

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## PROLONGED OR REPEATED COPULATION AND MALE LONGEVITY IN THE TICK *IXODES RUBICUNDUS*

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**ABSTRACT:** Male *Ixodes rubicundus* were found *in copula* with attached females in 59% of individuals. The number of males attaching to the female integument or to the host was negligible. In laboratory experiments, males enclosed with females in a small vial had significantly longer life spans than solitary males. Paired males were found to be *in copula* on 20-34% of observations. Solitary males lost weight while paired males gained weight over a 2-day period. However, the specific energy content of the 2 groups remained constant.

A male and a female found *in copula* are usually presumed to be mating, with the male fertilizing eggs produced by the female. A series of behaviours leads to copulation, with the male often soliciting the female for a chance to mate and the female taking an active part in choosing between suitors. Both sexes benefit from the resultant mating by producing offspring that carry their genetic endowment into the next generation. Ticks are among the many groups of animals showing this pattern of behaviour (Andrews, 1982; Sonenshine, 1985).

Ticks of the genus *Ixodes* have larval, nymphal, and adult life history stages, each of which requires a separate host to supply a blood meal. After feeding, ticks detach and fall to the ground where they moult to the next life history stage. The genus *Ixodes* differs from the general pattern of the hard ticks (Ixodidae), as spermatogenesis and initial stages of spermiogenesis take place in the late nymphal and early adult stages. Neither sex needs to feed before mating, which may take place on the ground or on the host (Oliver, 1982). However, the interaction between the sexes in *Ixodes* is not confined to mating. Males of many *Ixodes* species do not feed on a host but are frequently found parasitizing females, from which it is assumed they obtain haemolymph or host blood (Moorhouse, 1966; Norval, 1974; Moorhouse and Heath, 1975). Moreover, males and females may be found *in copula* for much longer periods than are necessary for spermatophore transfer (Stampa, 1959; Norval, 1974). Full engorgement occurs on the host and in most females it usually occurs only after mating (Diehl

et al., 1982). However, prolonged copulation in *Ixodes* frequently occurs when females are nearing full engorgement (Stampa, 1959; Norval, 1974). One may therefore question the reasons for such prolonged copulation as the more simple explanation of mating does not seem likely.

In this paper we discuss the influence of prolonged or repeated copulation on male *Ixodes rubicundus*.

### MATERIALS AND METHODS

*Ixodes rubicundus* adults were collected at weekly intervals from 20 individuals chosen from a flock of 60 sheep on the farm Preezfontein (29°50'S, 25°23'E) in the southern Orange Free State, South Africa. Collections were made from the first week of April to the last week of August 1986. Attached and unattached females, solitary males, and males *in copula* were counted and then placed individually in vials.

Male survival was determined both for solitary males and males that had been found *in copula*. In each of 3 separate trials, 10 solitary males were weighed and placed individually in 6.5-ml glass vials. Another 10 males that were found *in copula* were removed from the females and weighed prior to being placed in a vial in which a partially engorged female of known weight had been placed. The vials were plugged with cotton wool and maintained at ambient conditions of 13-23°C and 30-40% relative humidity. Where possible, observations were made daily to determine the survival of both solitary (control) males and males in the presence of females (experimental). The experimental males were also recorded as being *in copula* or not.

In another experiment, relative changes in the mass of males *in copula* were compared with those of solitary males over a 2-day period. All males were collected in the field by blanket dragging (Stampa, 1959), returned to the laboratory and kept at 95% relative humidity for 2 days, and then weighed. Fifteen males were then placed individually in 6.5-ml glass vials. Another 15 males were placed individually in 6.5-ml vials with a semiengorged female. The vials were kept under ambient conditions of temperature and humidity for 2 days. At the end of this time, males that had access to females but were not *in copula* were discarded, leaving 8 males in the experimental group. All remaining males

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TABLE I. Field data for adult *Ixodes rubicundus* collected from sheep. The number of males and females collected over weekly periods per month for each month of collection.

Month	Number of females	Number of males	Number of males			
			<i>In copula</i>	Solitary	Attached to female integument	Attached to host
April	203	136	70	66	0	0
May	1,643	1,090	610	480	3	0
June	907	333	250	83	0	2
July	627	209	117	92	0	3
August	174	27	11	16	0	0
Total	3,554	1,795	1,058	737	3	5

were weighed and then dried to a constant weight in a Speedi-vac freeze drier and reweighed.

Relative changes in the energy content of these 2 groups of males were determined using the method of van der Westhuizen et al. (1985). Saturated chromic acid was diluted 10 times with concentrated sulphuric acid. Each male was placed in 2 ml of this solution and digested for 30 min at 110 C. The digest was cooled and slowly diluted to twice its original volume with distilled water. Optical densities were determined on an LKB Ultrospec (model 4051) at 605 nm against a reagent blank. Glucose with an energy content of 2,872 kg/mole was used to standardize the method.

RESULTS

Adult *I. rubicundus* were collected from April to the end of August, with peak abundance occurring in May (Table I). Females consistently outnumbered males, with the degree of difference increasing as time elapsed. With the exception of August, when few adults were found, more than 50% of the males were *in copula*, with a maximum of 75% in June (Table I). Only in a few cases did males actually parasitize females or attach to the host (Table I).

The females with the experimental males were all semiengorged, weighing approximately 200 mg (Table II). A sample of 10 fully engorged females ranged from 282 to 340 mg with a mean

of 305 mg ( $\pm 7$  mg). A comparison of the 3 replicate groups for both control and for experimental males at the commencement of each experiment showed that although significant differences did occur in initial male weight between replicates (2-way analysis of variance,  $F_{2,54} = 31.56, P < 0.001$ ), no differences existed between control and experimental males ( $F_{1,84} = 2.44$ , not significant [n.s.]) and no interactions occurred ( $F_{2,54} = 1.33$ , n.s.).

Males in the presence of females survived for up to 8–16 days longer than control males, with a mean survival of 3.4–8.4 days longer (Table II). Male survival differed significantly between control and experimental groups (2-way ANOVA,  $F_{1,84} = 5.29, P < 0.01$ ) as well as between replicates (2-way ANOVA,  $F_{2,84} = 30.55, P < 0.001$ ). There were no interactions (2-way ANOVA,  $F_{1,84} = 3.07$ , n.s.). The length of time an individual male survived was significantly, positively correlated with the number of days on which that male was found *in copula* (Table II). This correlation accounted for 80%, 67%, and 93% of the variance ( $r^2_{xy}$ ) in the length of survival for replicates 1, 2, and 3, respectively.

Males that were left with females for 2 days and that were *in copula* at the end of that time

TABLE II. A comparison of survival times for *Ixodes rubicundus* males either with access to a female or solitary for 3 groups of ticks.

Experimental replicate	Date of commencement	Control/experimental	Initial female weight, mg ( $\pm$ SE)	Initial male weight, mg ( $\pm$ SE)	Time for all animals to die	Mean number of days surviving ( $\pm$ SE)	Mean number of days in copula ( $\pm$ SE)	$r_{xy}$ *
1	30 May 1986	Control		11.7 (0.82)	4	1.8 (0.34)	0	0.895
		Experimental	215.5 (31.8)	13.3 (0.80)	12	5.2 (1.33)	2.5 (0.52)	( $P < 0.001$ )
2	23 June 1986	Control		9.5 (1.00)	3	1.7 (1.28)	0	0.818
		Experimental	202.9 (52.0)	11.11 (0.70)	12	6.3 (1.28)	4.1 (1.13)	( $P < 0.01$ )
3	30 June 1986	Control		14.2 (0.30)	5	2.7 (0.37)	0	0.965
		Experimental	238.8 (58.0)	14.7 (0.40)	21	11.1 (1.96)	4.5 (1.25)	( $P < 0.001$ )

\* Pearson product moment correlations ( $r_{xy}$ ) are given with the significance levels relating male survival to the number of times males were found in copula. Sample size for all groups was 10.

TABLE III. Weight change in *Ixodes rubicundus* males kept solitary for 2 days compared with males that had access to females and that were found in copula at the end of 2 days.

	Number of males	Starting weight, mg ( $\pm$ SE)	Weight after 2 days, mg ( $\pm$ SE)	Dry weight, mg ( $\pm$ SE)	Specific energy content (J/mg)
Experimental group	8	1.07 (0.17)	1.46 (0.32)	0.52 (0.10)	12.43 (0.88)
Control group	15	1.06 (0.18)	0.91 (0.15)	0.45 (0.06)	12.82 (1.40)
<i>t</i> -value*		0.13 (n.s.)	5.74 ( $P < 0.001$ )	2.11 ( $P < 0.05$ )	0.71 (n.s.)

\* *t*-tests compare the starting weights of the 2 groups as well as the weight after 2 days, the dry weight, and specific energy content. n.s. = not significant.

gained weight (paired *t*-test comparing the same males on day 0 and day 2:  $t_7 = 3.71$ ,  $P < 0.1$ ). By contrast, control males weighed less over this period ( $t_{14} = 5.73$ ,  $P < 0.001$ ). The control group was significantly lighter than the experimental group (Table III). After drying, the experimental males were still significantly heavier than the controls, but the specific energy content of the 2 groups did not differ (Table III).

#### DISCUSSION

Within the genus *Ixodes* there are usually fewer males than females on the host (Yeoman and Walker, 1967; Norval, 1974; Walker, 1974), probably because mating often takes place on the ground (Arthur, 1962). Consequently, the sex ratio of the parasitic population does not reflect that of either breeding adults in the laboratory or of free-living adults collected by dragging (Fourie and Petney, unpubl. data). In other genera of ticks, where mating occurs on the host, the sex ratio is usually biased towards males (Yeoman and Walker, 1967; Walker, 1974).

The strategy of penetrating the female integument and (presumably) feeding on host blood or haemolymph is found in many members of the genus *Ixodes* (Moorhouse and Heath, 1975). In *Ixodes pilosus* (closely related to *I. rubicundus*), equal numbers of males have been found attached to a female's integument and *in copula* (24.1% each, Norval, 1974). Such females are often in an advanced stage of engorgement and an individual may have been parasitized many times (Moorhouse and Heath, 1975).

This is rare in *I. rubicundus*. However, the males found *in copula* weighed more and lived longer relative to males not found *in copula*. We hypothesize that the males are maintaining their water balance and deriving some nutritive benefit at the expense of the female. The lack of

significance between the groups in specific energy content is perhaps due to the overwhelmingly large quantities of structural body material going into the digest.

It is also possible, however, that males are able to benefit from the presence of the engorged female through the increased relative humidity in a small space. Petney (unpubl. data) has demonstrated that both male and female *Amblyomma hebraeum* (bont ticks) survive longer when aggregated in vials plugged with cotton wool than when alone. However, although water loss was reduced, no gain in weight was ever recorded. This hypothesis could be further tested by equalizing tick biomass in control and experimental groups by adding more males to the control group.

As in other members of the genus *Ixodes*, attachment either to the female integument or in copulation is found predominantly in females approaching full engorgement. As mating can occur on the ground and engorging females have thus probably already been fertilized, it seems unlikely that extended times *in copula* represent the act of mating. If our hypothesis is correct and the males are parasitic on the females, they would prolong their own life span and hence chances of future copulation. If the male *in copula* has not previously mated, parasitizing a female previously mated by a different male would seem an acceptable strategy for increasing the unmated male's chances of eventually mating.

The role of the female, in allowing the male to attain the copulation position, and whether the female is physically affected by the prolonged copulation are unknown.

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## BOOK REVIEW . . .

**Black Flies, Ecology, Population Management, and Annotated World List**, Ke Chung Kim and Richard W. Merritt (eds.). The Pennsylvania State University Press, University Park, Pennsylvania. 1988. 528 p. \$48.50.

Black flies (simuliids) are extremely important pests affecting human and other animals worldwide. They are extremely effective vectors of onchocerciasis and avian malaria. Because of their medical and economic importance, much has been published concerning the taxonomy, biochemistry, cytogenetics, ecology, and control of black flies. This book evolved from an international conference held in 1985 and attended by many of the world's foremost authorities. It integrates a wide range of research findings from a multidisciplinary perspective.

The book contains 32 chapters and is divided into 8 sections. Section 1 presents a comprehensive review of the functional role of black flies in stream ecosystems. Section 2 has 4 chapters that describe the current status of black fly systematics. The role of systematics in population management is discussed in a very useful way. Section 3 contains 15 chapters that deal with the ecology of immatures. These chapters describe the

problems associated with estimating populations, factors affecting distribution, hydrodynamics, and the sensory basis of behavior. Section 4 deals with population management. Its 5 chapters discuss control measures, maintaining black flies in the laboratory, and the ecology of black fly parasites. The 4 chapters that make up section 6 discuss the epidemiology and control of simuliid-born diseases. Section 7 contains 2 chapters that discuss the problem of black fly control from the industrial perspective. One chapter describes the use of Teknar®, a biological insecticide that contains delta-endotoxin crystals and spores of *Bacillus thuringiensis*. The second describes the use of Vectobac, a wettable powder formulation of *B. thuringiensis*. The problems associated with formulation improvement are discussed in detail. Section 8 is a very comprehensive annotated checklist of the world black flies.

This book brings together a vast amount of information that should prove to be most useful to researchers concerned with the biology, systematics, and control of simuliids. It is an excellent addition to our knowledge of these insect pests.

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## LABORATORY SELECTION OF *HAEMONCHUS CONTORTUS* FOR RESISTANCE TO IVERMECTIN

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**ABSTRACT:** The eighth generation of adult *Haemonchus contortus*, selected by subjecting infected pairs of sheep to suboptimal ivermectin treatment once per generation from parent (P; BBH isolate) through  $F_7$  (IV-A; selected isolate), required an approximate 4-fold increase in the ivermectin dose to produce 95% efficacy compared with its contemporary parent isolate. In a dose titration experiment the dose-response curve of the drug pressure-derived isolate, IV-A, was significantly ( $0.02 < P < 0.05$ ) less steep than was the response curve of the parent, BBH, isolate. Potency estimates based upon these nonparallel dose-response curves would not remain constant over a range of efficacy levels but would decrease rapidly at efficacies  $> 95\%$ . Passage of a closed population of the  $F_8$  generation of IV-A sequentially through pairs of sheep for an additional 11 generations ( $F_{8A}-F_{8K}$ ) without additional drug pressure being applied produced no reversion to sensitivity to ivermectin relative to the  $F_7$  generation, thus suggesting that the selected "resistance" was stable.

*Haemonchus contortus* is a ubiquitous nematode noted for its propensity to develop significant resistance to broad-spectrum anthelmintics. Other nematode parasites, such as *Trichostrongylus colubriformis*, *Ostertagia circumcincta*, and some of the small strongyles of equids and a number of arthropod parasites subjected to intense control by chemotherapeutic regimes have been known to become resistant to broad-spectrum agents as well.

Most recorded occurrences of altered sensitivity of nematode parasites to one or another anthelmintic have been recognized under field conditions of either commercial production or pastoral experimental situations. Relatively few have been recognized as the result of laboratory selection procedures. One study (Kates et al., 1973), which reportedly selected a "sensitive" isolate of *H. contortus* for cambendazole-resistance in 4 generations, is open to question as these authors in a prior publication (Colglazier et al., 1970) indicated that their *Haemonchus* isolate was "resistant" to thiabendazole to some degree, and cross-resistance of isolates among benzimidazoles within *H. contortus* is well known. The following is a description of the successful laboratory selection of an ivermectin-resistant isolate derived from an anthelmintically naive parent *H. contortus*.

### MATERIALS AND METHODS

The parent *H. contortus* used in this study was isolated in 1957 from a closed flock of sheep housed on an experimental farm in Branchburg Township, New Jersey. This isolate (Branchburg *Haemonchus* = BBH)

has been propagated via sequential passage in parasite-free lambs without exposure to anthelmintics of any description since that time. From about 1950 to 1957 the helminth population on Branchburg Farm had been exposed only occasionally and minimally to salvage treatments with phenothiazine. Egerton et al. (1964) reported that phenothiazine was 95% effective against this isolate at 446 mg/kg and that thiabendazole was 95% effective at 51 mg/kg, on average.

### Selection protocol

Sheep utilized during this study were raised parasite-free, having been moved indoors on day of lambing, and were from 2 to 10 mo old when infected. For each generation of parasites, infection was done in a pair of lambs, 1 wether and 1 ewe, using an infective dose of 10,000  $L_3$  per lamb. On the 21st, 23rd, and 25th days postinfection individual fecal samples were examined for quantitative estimates of the number of eggs of *H. contortus*. Ivermectin treatments were administered (usually on the 28th postinfection day) as single oral doses to each pair of lambs at 0.020 mg/kg. This dosage was chosen to produce  $\approx 95\%$  reduction in the number of *H. contortus* eggs passed posttreatment relative to the number passed pretreatment. Beginning 7 days posttreatment, and continuing for an additional 5-7 days thereafter, total fecal output was collected separately from each of the pair of treated lambs and incubated at 27°C and 90-95% relative humidity for 5-7 days to allow development of infective larvae.

The first adult generation to be subjected to ivermectin selection pressure in this experiment was designated the parent BBH ( $P_{BBH}$ ), and the larvae derived therefrom were designated IV-A  $F_1L_3$ . Five thousand  $F_1L_3$  from the wether lamb were combined with 5,000  $F_1L_3$  from the ewe lamb to produce the infective dose for each of the second pair of lambs, again 1 wether and 1 ewe, and so on for each subsequent generation. However,  $F_3L_3$  were obtained only from the wether lamb because the ewe lamb of the pair did not produce any viable *H. contortus* eggs following ivermectin treatment. Treatment of fifth-generation adults necessitated infection of a second pair of lambs because the ivermectin treatment in the first infected pair produced far greater reduction than anticipated from responses not-

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TABLE I. Mean efficacy of a single oral dose of ivermectin against the parent and IV-A F<sub>7</sub>-derived isolates of *Haemonchus contortus* at patency in experimentally infected sheep.

Treatment, mg/kg	Isolate					
	BBH (parent)			IV-A (F <sub>7</sub> )		
	Mean worm burden	% Efficacy		Mean worm burden	% Efficacy	
		Observed	Calculated*		Observed	Calculated*
0 (control)	4,380	—	—	3,257	—	—
0.009	1,098	75	59	—	—	—
0.016	337	92†	96	—	—	—
0.028	13	99.7‡	99.7	2,112	35	58
0.050	0	>99.9‡	99.9	292	91	91
0.090	—	—	—	15	99.5†	98
0.160	—	—	—	33	99.0†	99.6

\* Calculated from the linear regression of log<sub>10</sub>(worm count) on log<sub>10</sub>(dose) of Table II; i.e., the expected "average" responses over all observations within isolate.

†, ‡ Reduction from respective columnar control values due to treatment,  $P < 0.01$ , 0.001, respectively.

ed in earlier generations. The greater than expected response to ivermectin treatment in the first F<sub>3</sub>-infected pair of lambs could not be accounted for retrospectively as being due to, e.g., inadvertent overdosing.

#### Dose titration

A dose titration study to detect possible differences in response to ivermectin treatment was conducted simultaneously on the adult generation of IV-A F<sub>7</sub> and contemporary adult BBH. Forty-two young parasite-free sheep were divided into 2 equivalent groups, based on sex and body weight, of 21 sheep each. Each lamb in the first group was infected with 10,000 L<sub>3</sub> of the BBH parent isolate of *H. contortus* and each lamb in the second group was infected with 10,000 L<sub>3</sub> of the selected IV-A F<sub>7</sub> isolate. The inoculum for each lamb was concentrated in 5 ml tap water and delivered via syringe per os (p.o.). Each isolate group was then subdivided by restricted randomization based on sex and body weight into 5 treatment groups of 4 sheep per group; the median weight ewe of each isolate group was randomly assigned to 1 of the 5 treatment subgroups, which was then designated as the infected (placebo-treated) control group. The 4 remaining treatment groups within each isolate were randomly assigned to ivermectin treatment thus: (a) for BBH, dosages of 0.009, 0.016, 0.028, or 0.050 mg/kg, p.o., and

(b) for IV-A F<sub>7</sub>, dosages of 0.028, 0.050, 0.090, or 0.160 mg/kg, p.o. Treatments, including placebo vehicle, were administered to individually weighed sheep as a single oral dose at a volume of 0.1 ml/kg of body weight on the 22nd day postinfection. The sheep were killed for recovery of residual worm burdens 7–8 days posttreatment. General methods for infection, treatment, and necropsy were as described in Egerton et al. (1979). All mean worm counts were recorded as geometric means because common log transformations were used for analyses of worm counts and dose levels through analysis of variance and linear regression methods. A sample of the ivermectin solution used was sent for assay of ivermectin content following treatment of the IV-A F<sub>7</sub> adult infections.

#### RESULTS

Fecal worm egg counts for the P, F<sub>1</sub>, F<sub>2</sub>, and F<sub>3</sub> generations of *H. contortus*, each selected with a single oral dose of ivermectin at 0.02 mg/kg, were reduced 93.6% on average following treatment. Reduction in worm egg counts following treatment of the F<sub>4</sub>- and F<sub>5</sub>-generation adults was only 21.7% on average, whereas for the F<sub>6</sub> and F<sub>7</sub> generations no decrease in average worm eggs per gram of feces (EPG) occurred.

Analysis of variance performed on the log-transformed worm counts for the individual infected control sheep of both isolates indicated no difference in the mean sample worm populations ( $P = 0.662$ ); the infectivity of both isolates was thus equivalent. A loss in sensitivity of the IV-A F<sub>7</sub> isolate to ivermectin treatment was clearly suggested, relative to its BBH parent stock, on the basis of the data in Table I. The analyses of variance on the log-transformed worm counts, within isolate, and Duncan's multiple range tests are summarized in Table I along with calculations of the observed efficacies. Comparison of these responses within the 2 common dose levels (0.028 and 0.050 mg/kg) again reveals the decreased efficacy for ivermectin versus the IV-A isolate. Regression analyses of the log-transformed worm counts on the log-transformed dose levels, within isolates, demonstrated that the

TABLE II. Dose response of BBH and IV-A F<sub>7</sub> isolates of *Haemonchus contortus* to a single oral dose of ivermectin in experimentally infected sheep.

<i>H. contortus</i> isolate	Regression equation*	ED <sub>95</sub> ± SE† mg/kg	At the 95% effective dose (ED <sub>95</sub> )	
			Relative ivermectin potency	Ivermectin resistance factor
BBH (parent)	$\log \hat{Y} = -4.236 \log X - 5.4098$	0.015 ± 0.004	1.0	1.0
IV-A (F <sub>7</sub> )	$\log \hat{Y} = -2.642 \log X - 0.9720$	0.062 ± 0.039	0.24	4.13

\*  $\hat{Y}$  = predicted mean worm burden;  $X$  = dosage of ivermectin, mg/kg. Student's  $t$ -test of regression coefficients showed the above dose-response curves to be nonparallel,  $0.02 < P < 0.05$ ; both regression coefficients different from zero,  $P < 0.05$ .

† By Cochran's approximation, a 1-term Taylor expansion.



dose-response curves of the 2 isolates were not parallel ( $0.02 < P < 0.05$ ) and that the potency of ivermectin against the IV-A  $F_7$  isolate was only  $0.24 \times$  its potency against the parent BBH isolate's 1.0, at their respective 95% effective dose ( $ED_{95}$ ) level (Table II). Because of nonparallel dose-response curves, potency estimates will vary, depending upon which efficacy level(s) is (are) chosen for comparison, but these data show that, at the respective  $ED_{95}$ 's, IV-A  $F_7$  was  $4.13 \times$  less responsive than was BBH (and the discrepancy would be even greater for efficacies  $>95\%$ ).

The individual worm counts for the IV-A  $F_7$  isolate at dose levels 0.090 and 0.160 mg/kg (not shown) suggested that responses for this selected isolate were somewhat variable even at reasonably high "average" efficacy values. The inference was confirmed by the fit of the expected efficacy calculated from the regression equation relative to the observed data for each isolate as displayed in Table I.

The ivermectin solution used for the selection treatments (containing a nominal 0.4 mg ivermectin/ml) was found by analysis to contain 0.43 mg ivermectin/ml (J. R. Zingerman, pers. comm.), which was within nominal expectation indicating no loss in potency of the formulation used.

## DISCUSSION

The results of the dual titration study have demonstrated that a mechanism for resistance to ivermectin was selected in *H. contortus* by administration of a single oral dose of ivermectin once per generation for not more than 7 generations at a dose level initially producing an approximate 95% reduction in the infecting parent population.

The parent population (BBH) was purposely chosen for having been removed from any exposure to either narrow or broad-spectrum anthelmintics for at least 25 yr prior to the application of selective pressure with ivermectin. Although BBH was "anthelmintically naive" it was not a truly "wild type," having been serially passaged as a closed population at least twice per year for each of the preceding 25 yr. Arguably, the choice of starting with BBH rather than some recent field isolate with polyvalent resistance to, say, benzimidazoles, levamisole, and pyrantel/morantel may not have been an optimum procedure. One could propose that recently isolated polyvalent resistors would have their "survival mechanism" gene pool primed and activated. A

more cogent argument, in our opinion, is that the gene frequency for "resistance" to a given toxic agent is finite and fixed in any target population. This is to be expected regardless of prior exposure or current state of "resistance" and it is thus merely an exercise in patience to "select" for resistance given an appropriate selection dose level, sufficient animal resources, and a closed target population.

The mechanism of this resistance to ivermectin remains unknown as biochemical attempts to measure differences in uptake, binding affinity, etc., have yet to be successful.

Another question arising with each recorded instance of anthelmintic resistance deals with the permanence of the condition: is resistance permanent within a closed population or will it regress toward parental sensitivity over time? While maintaining an absolutely closed population through strict sanitary measures, the  $F_8$  generation of the IV-A isolate was sequentially passaged through 11 successive pairs of sheep for 11 generations ( $F_{8A}$ – $F_{8K}$ ) without any drug pressure, then tested for ivermectin resistance via dose titration. The  $F_{8K}$  IV-A adult *H. contortus* were reduced in numbers by 95% with an ivermectin dose level of  $\approx 0.060$  mg/kg, which is well within the originally estimated range for IV-A  $F_7$  (Table II:  $0.062 \pm 0.039$  mg/kg). It would appear reasonable to deduce that IV-A ivermectin resistance is relatively stable.

The rate of resistance selection (i.e., the number of selection generations required to effect a significant shift in drug sensitivity) is highly dependent upon (i) the frequency of occurrence of the gene(s) for resistance in the population at risk (the frequency could be variable in disparate selected populations), (ii) variation in the way an anthelmintic is utilized in the laboratory, as opposed to the field, and (iii) the relationship between the "selective" drug dose and the dose actually applied (which may not be the manufacturer's recommended dosage). Examining premise (i), and given a sufficiently large number of sheep each infected with a sufficiently large number of *Haemonchus*, it is theoretically possible to select for a genetically less sensitive subpopulation from the population at risk through application of a single "high" anthelmintic dose under conditions of no dilution of the residual resistant worms. In a large population even low frequency "resistant genes" could be brought forth in a single "selective" event provided  $<100\%$

efficacy was attained and some breeding population survived in the treated sheep.

Although we know that resistance to broad-spectrum anthelmintics does occur, there are relatively few data regarding the rate at which selection for resistance occurs in *H. contortus*. Kates et al. (1973) did report a laboratory study purporting to have selected a cambendazole-resistant *H. contortus* after applying drug pressure for only 4 generations (at 5, 5, 10, and 20 mg/kg for P, F<sub>1</sub>, F<sub>2</sub>, and F<sub>3</sub> generations, respectively). These same authors (Colglazier et al., 1970) had previously described their 1973 "cambendazole sensitive" BPL-2 isolate of *H. contortus* as having "some degree of tolerance to thiabendazole at . . . 50 mg/kg . . ." In fact thiabendazole produced only 67% efficacy against a 3-wk-old infection of BPL-2 at 50 mg/kg (Colglazier et al., 1970). A thiabendazole-sensitive adult *H. contortus* population would be expected to be reduced by  $\approx 95\%$  by thiabendazole at 50 mg/kg (Egerton et al., 1964).

Although a measurable resistance to cambendazole was selected for in BPL-2 *H. contortus* in 4 generations (Kates et al., 1973), it is difficult to assess whether some apparent prior exposure of their isolate to thiabendazole (Colglazier et al., 1970) may have increased the subsequent rate of selection with cambendazole. The above, plus the results of the experiments reported here, indicate that selection for resistance to anthelmin-

tics in *H. contortus* may occur in as few as 4, but certainly not more than 7, generations exposed to selective drug pressure.

#### ACKNOWLEDGMENT

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## SCHISTOSOMA MANSONI AND S. JAPONICUM WORM NUMBERS IN 129/J MICE OF TWO TYPES AND DOMINANCE OF SUSCEPTIBILITY IN F<sub>1</sub> HYBRIDS

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**ABSTRACT:** In a study on the genetics of resistance to schistosomiasis in WEHI 129/J mice, susceptibility to either *Schistosoma mansoni* or *Schistosoma japonicum* was shown to be unequivocally dominant in F<sub>1</sub> hybrid crosses between genetically resistant WEHI 129/J and susceptible BALB/c mice. The operation of only 1 or 2 genes in the expression of resistance to *S. mansoni* was suggested by backcross analysis. Thus, approximately 25% of (BALB/c × WEHI 129/J) F<sub>1</sub> × WEHI 129/J mice were resistant to *S. mansoni* infection, whereas resistance was manifest in approximately 50% of WEHI 129/J mice. The data are consistent with resistance being controlled by 1 recessive gene having 50% penetrance. We also report that 129/J mice obtained directly from the Jackson Laboratories (Bar Harbor, Maine) (designated JAX 129/J), differ from locally bred WEHI 129/J in being entirely susceptible to *S. mansoni* infection. However, both WEHI 129/J and JAX 129/J are relatively resistant to *S. japonicum* infection.

It was reported previously that 129/J mice bred at this Institute (designated WEHI 129/J) were resistant to chronic infection with *S. japonicum* because a high proportion rejected worms (Garcia et al., 1983b; Mitchell et al., 1984). The proportion of resistant mice in most experiments is approximately 50%. This same resistance to infection is found using *S. mansoni* (Tiu et al., 1986). Moreover, WEHI 129/J mice sent to other laboratories have proven to be relatively resistant to schistosomiasis *mansoni* so there is no doubting the peculiarity that exists in these mice (Bout, pers. comm.; Elsaghier Knopf and McLaren, pers. comm.). However, Fanning and Kazura (1984) reported nothing unusual about 129/J mice with regard to *S. mansoni* infection. The findings suggest that a variation has arisen in the locally bred WEHI 129/J compared to stock 129/J mice. In confirmation of the results of Fanning and Kazura, we report here that 129/J mice purchased from the Jackson Laboratories, Bar Harbor, Maine (JAX 129/J), are entirely permissive to *S. mansoni* infection and thus differ from WEHI 129/J mice in this regard. Surprisingly however, JAX 129/J mice are more resistant to *S. japonicum* than they are to *S. mansoni* and resemble WEHI 129/J in their relative resistance to schistosomiasis *japonica*.

Resistance to schistosomiasis in WEHI 129/J

mice may be related, at least in part, to increased responsiveness of these mice to parasite glutathione S-transferase (GST) enzyme(s) (Mitchell, 1986; Smith et al., 1986). However, an involvement of additional factors is suggested by the failure to detect any increased or accelerated antibody response to *S. japonicum* GST's in the resistant proportion of WEHI 129/J mice (Davern et al., 1987). One powerful approach to elucidating immune responses essential for resistance is to correlate the inheritance of such responses with the inheritance of resistance (Erllich et al., 1983). In a first step to applying this approach to the WEHI 129/J model we present here the results of a study on the genetics of resistance to schistosomiasis in WEHI 129/J mice.

### MATERIALS AND METHODS

#### Mice

Male and female WEHI 129/J, BALB/c, and (BALB/c × WEHI 129/J) F<sub>1</sub> were produced in a specific pathogen-free facility but maintained conventionally from 6 wk of age. Additional 129/J mice (JAX 129/J) were purchased from the Jackson Laboratories and were maintained and bred under conventional conditions as were the backcross mice (BALB/c × WEHI 129/J) F<sub>1</sub> × WEHI 129/J. Mice were between 6 and 34 wk old (generally 8-17 wk) when first used for infection.

#### Parasites and infections

The *S. mansoni* (Puerto Rico) and *S. japonicum* (Philippines) parasites were maintained at the Hall Institute from stock formerly housed by one of us (J.C.W.) in the Sydney University School of Public Health and Tropical Medicine. Parasites were passaged through mice (and occasionally rabbits in the case of *S. japon-*

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icum), eggs being obtained from feces or, more usually, livers. Snails were maintained in a room of constant temperature (26–29°C) in aerated tanks of autoclaved purified water. Albino *Biomphalaria glabrata* were fed mainly on washed cos (romaine) lettuce, with occasional supplements of fish food flakes, a small amount of mouse food pellets (Barastoc Irradiated Feed, Melbourne, Australia), and blackboard chalk as a calcium source. They were treated periodically with 10% ethanol to reduce rotifers. Egg masses at the time of transfer to fresh containers were treated similarly in an attempt to also reduce *Chaetogaster* contamination. *Oncomelania hupensis quadrasi* were maintained in aerated tanks with minimal water over marble chips, rotting deciduous leaves, and chalk. These snails were provided with a supplement of filter paper soaked in skim milk and air dried. With the exception of infected *B. glabrata* likely to be shedding cercariae, the snails were exposed to bright fluorescent lights for approximately 10 hr (on at least 5 days per week) in addition to room lighting set at 14 hr light, 10 hr dark.

*Schistosoma mansoni* cercariae were shed periodically from 40 days of infection and *S. japonicum* cercariae were dissected from snails (at approximately 70 days infection) prior to application to the shaved abdominal surface of mice anaesthetized with Saffan (Glaxo Australia Pty Ltd., Boronia, Victoria) using a standard coverslip method (Garcia et al., 1983a). For *S. mansoni*, 100 cercariae were used but for the more lethal *S. japonicum* infection, 25 cercariae were applied to the skin.

Worm burdens were determined after 4–14 wk of infection (generally 7 wk). Mice were injected with 50 units of heparin, killed by CO<sub>2</sub> asphyxiation, and worms recovered by perfusion using either citrate saline or mouse tonicity phosphate-buffered saline pH 7.3, injected into the thoracic aorta and superior vena cava after cutting the portal vein. In several experiments, mice were bled every 10 days or so for analysis of antibody specificities in resistant and susceptible mice.

## RESULTS

### *Schistosoma mansoni* infection

In 5 independent experiments, male and/or female WEHI 129/J, JAX 129/J, BALB/c, or (BALB/c × WEHI 129/J) F<sub>1</sub> mice were exposed percutaneously to 100 cercariae of *S. mansoni* and worm burdens assessed 30–100 days later. Results of 3 experiments in which worm numbers were determined at 7–8 wk of infection are shown in Table I and combined results of the 5 experiments are presented in Figure 1. The data establish that approximately 50% of WEHI 129/J mice are resistant to chronic infection, this proportion being closer to 80% in results published previously (Tiu et al., 1986). In the *S. japonicum* system the average number of resistant mice in numerous experiments was 50% (Garcia et al., 1983b, and data presented below). In marked contrast to the WEHI 129/J mice exposed to *S.*

TABLE I. Susceptibility of JAX 129/J, BALB/c, and (BALB/c × WEHI 129/J) F<sub>1</sub> mice and resistance of WEHI 129/J mice to infection with *Schistosoma mansoni*.

Expt. no.*	Mouse strain (sex)	No. of mice	% Negative mice†	Mean worm burden in positive mice‡
1	WEHI 129/J (F)	8	50	21.3 ± 3.0
	BALB/c (F)	8	0	31.5 ± 3.3
	F <sub>1</sub> (F)	8	0	24.8 ± 6.9
2	WEHI 129/J (M)	13	46	23.7 ± 6.9
	JAX 129/J (M)	14	0	26.7 ± 1.7
3	WEHI 129/J (M)	7	57	12.3 ± 2.9
	F <sub>1</sub> (M)	7	0	14.7 ± 2.5
	JAX 129/J (M)	9	0	9.2 ± 1.4

\* Mice were 6–11 wk old in Experiment 1, 17–23 wk in Experiment 2, and 10–34 wk in Experiment 3. All mice were exposed to 100 cercariae and perfused 50, 49, and 54 days later.

† Mice with no detectable worms.

‡ Mice with ≥ 1 worm.

*mansoni*, the JAX 129/J mice are susceptible and resemble BALB/c mice (and all other mouse strains tested to date). F<sub>1</sub> hybrids between the susceptible BALB/c and resistant WEHI 129/J parental strains are completely susceptible.

Results of a backcross analysis are shown in Figure 2. Of 40 male and female (BALB/c × WEHI 129/J) F<sub>1</sub> × WEHI 129/J backcross mice exposed to 100 cercariae of *S. mansoni*, approximately a quarter were resistant in that they contained no or very few worms 9 wk later. Again in this experiment, control F<sub>1</sub> mice were susceptible and a proportion of WEHI 129/J mice were resistant. If the overall resistance of WEHI 129/J is close to 50% then the 25% resistance in a backcross population is consistent with unigenic genetic control of resistance, the gene having 50% penetrance. At most, 2 genes are likely to be operative.

### *Schistosoma japonicum* infection

In 2 experiments, female WEHI 129/J, JAX 129/J, BALB/c, and (BALB/c × WEHI 129/J) F<sub>1</sub> mice were exposed percutaneously to 25 cercariae of *S. japonicum* and worm burdens assessed 6–7 wk later. Results are presented in Figure 3. A high proportion of WEHI 129/J mice are resistant and worm burdens are comparable in the susceptible strain BALB/c (Garcia et al., 1983b; Mitchell et al., 1984) and F<sub>1</sub> mice with all mice containing worms. Thus, as in the case of infection with *S. mansoni*, susceptibility to *S. japonicum* is dominant in the cross between BALB/c and the resistant WEHI 129/J strains.

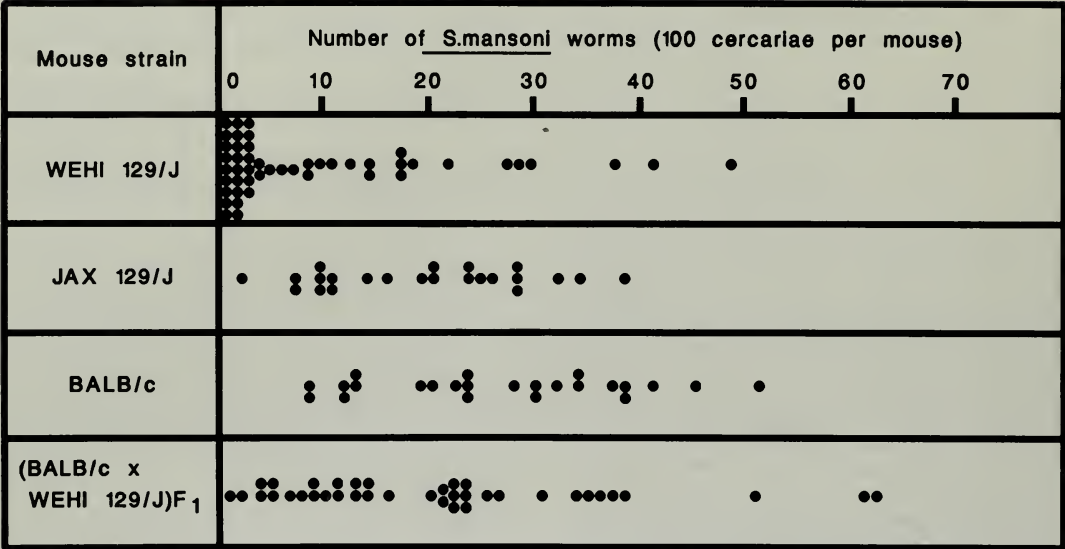


FIGURE 1. Number of worms recovered from 6–34-wk-old male and female mice of various strains infected with 100 cercariae of *S. mansoni* in 4 independent experiments (5 determinations). Worm burdens were determined at 4–14 wk of infection. The proportion of WEHI 129/J mice with <2 worms is 23/48.

In previous studies, the question of dominance of resistance/susceptibility to *S. japonicum* in F<sub>1</sub> mice was unresolved though again tending towards dominance of susceptibility (Garcia et al., 1983b). It is also apparent by comparing Figures 1 and 3 that *S. japonicum* infections in JAX 129/J are quite different from *S. mansoni* infections in this mouse strain; a significant proportion of JAX 129/J mice exposed to *S. japonicum* cercariae contain no worms several weeks later.

DISCUSSION

The key points to emerge from the studies reported here are (1) susceptibility of BALB/c mice is dominant over resistance in WEHI 129/J mice in F<sub>1</sub> hybrids between the 2 parental strains in both *S. mansoni* and *S. japonicum* infections, and (2) a single gene may operate in determining resistance to infection in WEHI 129/J mice, at least in the *S. mansoni* system (backcross studies have yet to be performed in the *S. japonicum* system). Another observation that confirms previous studies by others (Fanning and Kazura, 1984) is that JAX 129/J mice are entirely susceptible to *S. mansoni* infection. Yet they are relatively resistant to *S. japonicum* infection. This raises the question of what mutation(s) might have occurred in 129/J mice that results in the phenotype of partial resistance in schistosomiasis.

The breeding stock of the WEHI 129/J mice was originally obtained from the Jackson Laboratories some 16 yr ago, and thus the purchased JAX 129/J and the locally bred WEHI 129/J mice are likely to be very closely related. This should facilitate the identification and analysis of any immune responses that correlate with the resistant phenotype (Mitchell et al., 1984, 1985), or the susceptible phenotype for that matter (Butterworth, 1987). It is conceivable that WEHI 129/J mice fail to produce blocking antibodies (Butterworth, 1987; Capron et al., 1987), the capacity to do so in susceptible strains of mice being inherited as a dominant trait. Unigenic genetic control of such a response would seem unlikely.

With the assumption that immunology is involved at all in resistance or susceptibility to *S. mansoni* and *S. japonicum* in 129/J mice, the dominance of susceptibility suggests that self tolerance may be operating in the genetically susceptible mouse strains. Thus, a sharing of epitopes between a host-protective antigen in the schistosome and some molecule(s) in the susceptible but not the resistant mouse genotypes would account for the dominance of susceptibility to infection.

As referred to above, we have previously identified immune responses to a parasite GST as worthy of study with regard to the expression of



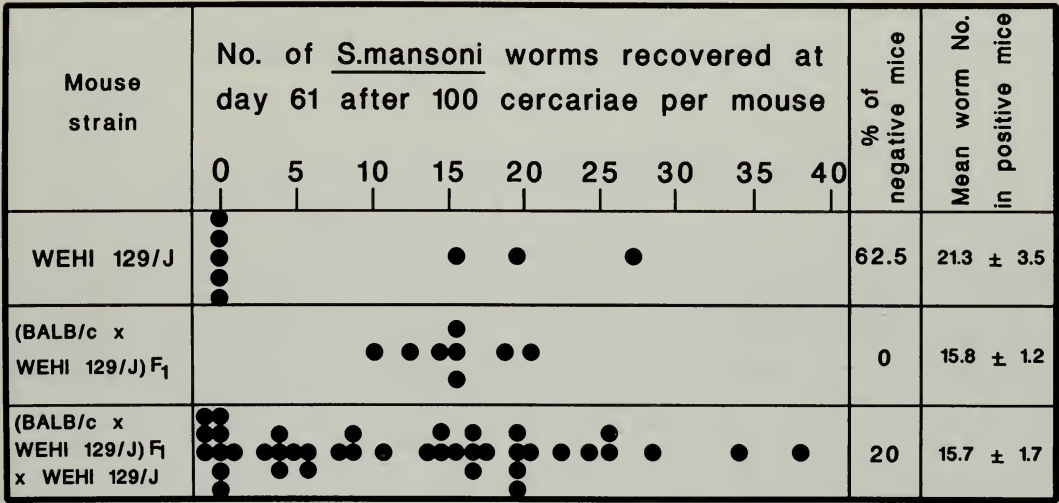


FIGURE 2. Number of worms recovered from 7–9-wk-old female WEHI 129/J and (BALB/c × WEHI 129/J) F<sub>1</sub> and male and female (BALB/c × WEHI 129/J) F<sub>1</sub> × WEHI 129/J backcross mice infected with 100 *S. mansoni* cercariae. Worm burdens were determined at 9 wk of infection.

resistance in WEHI 129/J mice. This emphasis stemmed from the early demonstration in the *S. japonicum* system that WEHI 129/J mice, unlike BALB/c mice, were high responders to an M<sub>r</sub> 26,000 soluble protein of adult worms (Mitchell et al., 1984, 1985) termed Sj26 and subsequently shown to be a GST (Smith et al., 1986). WEHI 129/J mice are also the highest IgG antibody responders of a range of mouse stains tested, following immunization with Sj26 produced in *Escherichia coli* (Davern et al., 1987). Both *S. mansoni* and *S. japonicum* worms contain 2 GST's, or at least GST subunits of M<sub>r</sub> 26,000 and M<sub>r</sub> 28,000 (termed Sj26, Sm26, Sj28, and

Sm28) (Davern et al., 1987). Comparing sera from WEHI 129/J, BALB/c, and JAX 129/J in protein A-immunoprecipitation studies using worm GST's labeled with the Bolton and Hunter reagent, responsiveness to Sj26 correlates with the resistant genotype in the *S. japonicum* system, whereas high responsiveness to Sm28 plus Sm26 may correlate with the resistant genotype in the *S. mansoni* system. However, these associations, particularly for *S. mansoni*, remain tentative and more comprehensive analyses are required.

Responses in F<sub>1</sub> mice have to date only been analysed in regard to anti-Sj26 antibodies and the picture is far from clear: during infection with

## RESISTANCE OF 129/J MICE TO *Schistosoma japonicum*

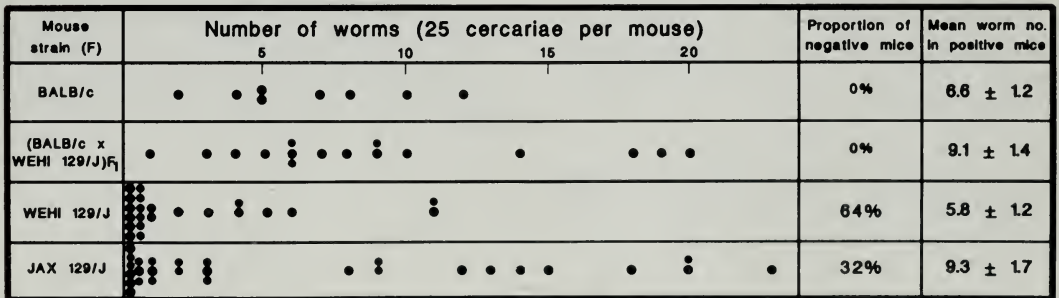


FIGURE 3. Number of worms recovered in 7–20-wk-old female mice of various strains infected in 2 independent experiments with 25 cercariae of *S. japonicum*. Worm burdens were determined at 7 wk of infection.



*S. japonicum*, F<sub>1</sub> mice are poor responders to Sj26 (Wright, unpubl. data), yet when injected with the functional native Sj26 produced in *E. coli*, and with Freund's complete adjuvant, they are good responders (Davern et al., 1987). A full analysis of correlates between anti-GST antibody responses (i.e., titers and isotypes) and resistance is in progress.

Some comment is warranted on the "variable resistance" of WEHI 129/J in both the *S. mansoni* and *S. japonicum* model systems. Although no evidence of residual heterozygosity exists in the WEHI 129/J mice (they type as 129/J mice on the basis of a panel of liver isoenzymes), the 50% resistance found on average over a large number of independent experiments is somewhat suspicious. Attempts are now being made to breed for resistance from repeatedly exposed WEHI 129/J mice. However, a likely explanation assuming genetic homogeneity in these mice is that a mutation has occurred in such mice either in an immune response gene or in a gene encoding a molecule sharing epitopes with a host-protective schistosome antigen (see above). If immune responses induced are not of high titre, with very few helper T cells perhaps being involved (Davern et al., 1987), variability in responsiveness early in the infection will result in variable worm burdens. The vulnerability of worms is apparently expressed during a period of schistosome development—this being apparently in the post-lung, pre-egg-laying period (Garcia et al., 1983b; Davern et al., 1987; El-saghier Knopf and McLaren, pers. comm.). Obviously, more detailed studies on the time and site of attrition of worms are needed before further speculation is justified.

#### ACKNOWLEDGMENTS

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## TWO HUMAN CASES OF GNATHOSTOMIASIS AND DISCOVERY OF A SECOND INTERMEDIATE HOST OF *GNATHOSTOMA NIPPONICUM* IN JAPAN

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**ABSTRACT:** Two human cases of gnathostomiasis from ingestion of raw native Japanese loaches, *Misgurnus anguillicaudatus*, are reported. Seven early third-stage larval *Gnathostoma nipponicum* were recovered from 3,098 loaches in the same district in which 2 human patients had obtained and eaten raw loaches. Encapsulated *G. nipponicum* larvae were also recovered from loaches infected under laboratory conditions. All 6 weasels captured in the same district in which the naturally infected loaches were found and where the humans had become infected were infected with adult worms of the same species. This is the first report of *M. anguillicaudatus* serving as a second intermediate host of *G. nipponicum*.

Human gnathostomiasis is a well-known zoonotic disease caused by nematodes of the genus *Gnathostoma*. Almost all of the Japanese human cases in the past have been associated with *Gnathostoma spinigerum*. Since 1980, however, there has been a considerable increase in the number of human gnathostomiasis cases in Japan (Demitsu and Aizawa, 1985), presumably caused by ingesting raw loaches, *Misgurnus anguillicaudatus*, imported from southeast Asia. Larval *G. hispidum* Fedtschenko, 1872, were recovered from the viscera of these loaches (Akahane et al., 1982), but strangely no *G. hispidum* have been found yet in human patients.

We have recently treated 2 human gnathostomiasis from ingestion of raw native Japanese loaches, *M. anguillicaudatus*. The brief case reports are as follows.

### Case 1

A 55-yr-old man from Ueno City in Mie Prefecture, Honshu, Japan, was admitted to the Mie University Hospital in July 1986 because of skin complaints. He ate a raw loach in May from a rice field in Ueno City. Two weeks later he noticed itchy, fleeting erythematous patches, and creeping eruption on his abdomen. The most recent erythematous patch was excised, sectioned, and stained with hematoxylin and eosin. One section of the tissue contained a part of a worm identified as a species of *Gnathostoma* (Fig. 1).

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### Case 2

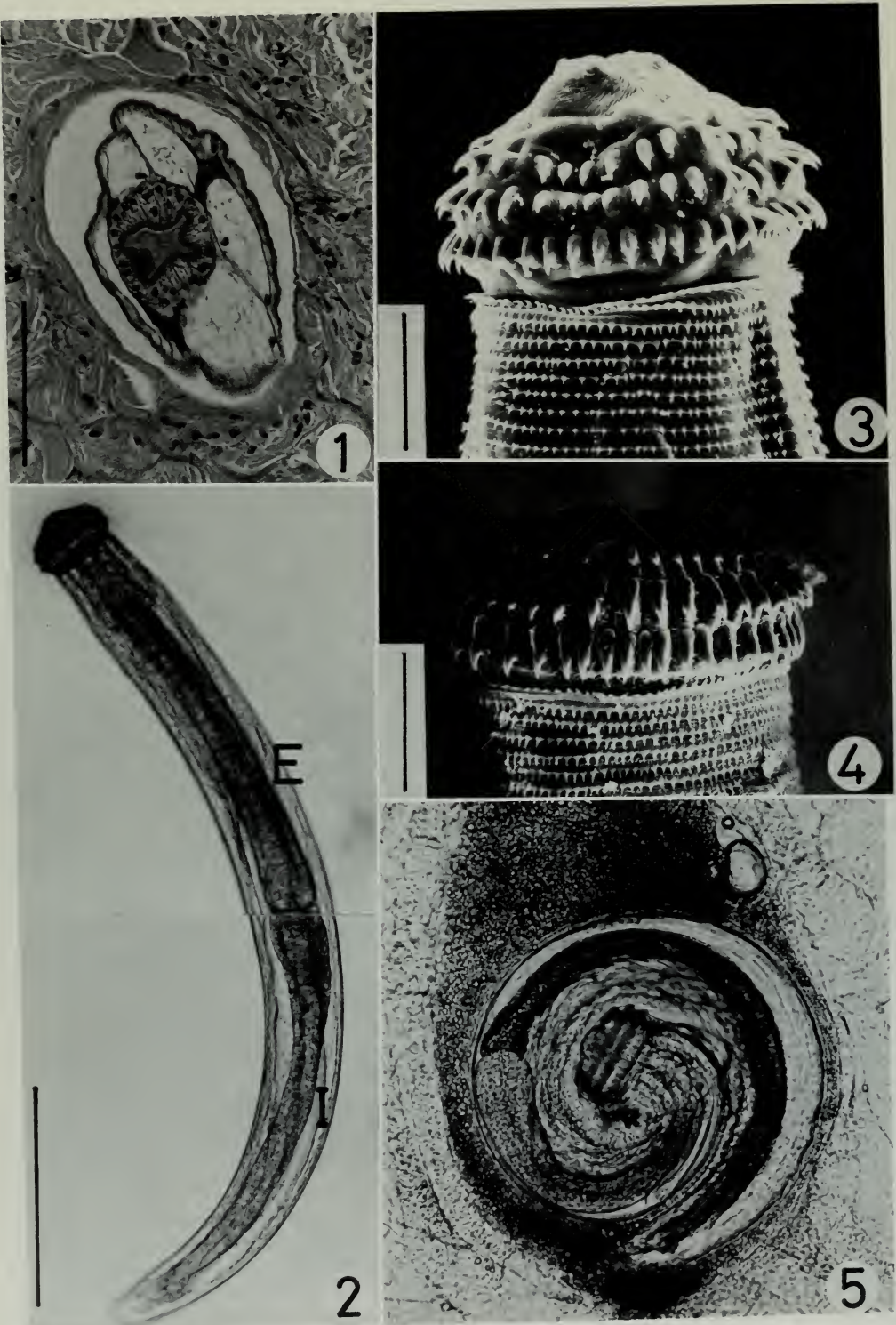
A 57-yr-old man from the same city as case 1 was admitted to Mie University Hospital in July 1986 because of skin complaints. He also had eaten 3 raw loaches in July, captured in Ueno City, but at a different location from case 1. Five days later he noticed the same clinical signs as noted in case 1. Samples of skin for histological examination were excised 3 times but no nematodes were found. Mebendazole, 300 mg per day, was given for 3 days and skin complaints disappeared within 2 wk. This case was diagnosed by an indirect fluorescent antibody test and Ouchterlony's test as positive for gnathostomiasis.

In order to survey causative agents of gnathostomiasis, loaches captured in Ueno City were examined and larval *G. nipponicum* were recovered from them.

Three species of *Gnathostoma* are native to Japan, i.e., *G. spinigerum* Owen, 1836, *G. doloresi* Tubangui, 1925, and *G. nipponicum* Yamaguchi, 1941. Adult *G. nipponicum*, a common parasite in Japan and only reported from there, is found in esophageal tumors of weasels.

Detailed morphological features of adult *G. nipponicum* were described by Yamaguchi (1941) and later Miyazaki and Umetani (1950), but little is known about the life cycle. Arita (1953), Isobe (1956, 1965), and Isobe et al. (1958) examined various vertebrates for larval stages of this nematode with negative results. Under laboratory conditions, Arita (1953) and Mabuchi (1956) demonstrated that second-stage larvae emerging from eggs were infective to 3 species of cyclopoid copepods, the first intermediate host. The larval nematode molted once in the copepods. At-





FIGURES 1-5. 1. Transverse section of worm in human tissue identified as belonging to the genus *Gnathostoma*. Bar = 100  $\mu$ m. 2-4. Early third-stage larvae of *G. nipponicum* recovered from loach captured in the field. 2. Larva from muscle. E = esophagus, I = intestine. Bar = 200  $\mu$ m. 3. Head bulb and cuticular spines of larva



TABLE I. Measurements (in  $\mu\text{m}$ ) of larval *G. nipponicum* recovered from naturally or experimentally infected loaches.

Source	Larva no.	Body length $\times$ width	Length of esophagus	Length of cervical sac	Head bulb length $\times$ width	No. of hooklets on head bulb			Transverse rows of cuticular spines on body	Place of recovery
						I	II	III		
Natural infection	1	410 $\times$ 55	295	150	30 $\times$ 53	32	36	39	<214	Viscera
	2	656 $\times$ 74	257	168	39 $\times$ 63	32	36	41	225	Viscera
	3	780 $\times$ 105	460	225	30 $\times$ 80	33	35	38	—	Muscle
	4	890 $\times$ 85	375	250	40 $\times$ 75	32	34	39	—	Muscle
	5	930 $\times$ 100	—*	—	33 $\times$ 90	34	36	38	<215	Muscle
	6	1,040 $\times$ 110	—	—	40 $\times$ 90	35	37	43	222	Muscle
	7	1,100 $\times$ 100	—	275	38 $\times$ 95	36	39	45	235	Muscle
Experimental infection	Means†	1,161.0 $\times$ 92.5	387.8	197.5	46.2 $\times$ 82.0	37.0	37.1	41.0	214.6	Muscle
	Ranges	Max. 1,780 $\times$ 135	580	300	60 105	38	41	43	225	
		Min. 700 $\times$ 60	280	140	35 60	35	37	40	203	

\* Indicates nonmeasurement by the disintegration of a portion of larva.  
 † Ten larvae from day 9 to day 100 postinfection.

tempts to infect fishes, amphibians, and reptiles with nematode larvae from copepods failed. Koga and Ishii (1981), however, recovered larvae from snakes and suggested that they might be paratenic hosts of *G. nipponicum*.

We report here the larval form of *G. nipponicum* recovered from loaches, *M. anguillicaudatus*, and 2 cases of human gnathostomiasis probably caused by *G. nipponicum*.

## MATERIALS AND METHODS

### Investigation of *G. nipponicum* in the field

Three thousand ninety-eight loaches, *M. anguillicaudatus*, were captured from July 1986 to April 1987 in Ueno City, Mie Prefecture, Japan. The muscles and viscera were dissected and digested with artificial gastric juice in attempts to find larvae. Recovered nematodes were fixed in 10% formalin and washed in a phosphate buffer prior to postfixation in 1% osmium tetroxide for 2 hr. Then they were dehydrated in a graded series of ethanol, dried in a critical-point drying apparatus, and coated with gold in a JFC-1100 ion-sputtering apparatus. Observations were made with a JSM-T200 scanning electron microscope operated at 10 kV.

Six weasels, *Mustela sibirica itatsi* and *M. s. coreana*, from the same geographical area as that containing infected loaches, were examined during the winter of 1987 for the presence of adult gnathostomes.

### Experimental infection of loaches

Two captured weasels with eggs of *G. nipponicum* in the feces were maintained in the laboratory. The eggs were washed 5 times with water and maintained

in water at 30 C for 6 days. The copepods (*Cyclops vicinus* and *Thermocyclops hyalinus*) collected in Tsu City were allowed to feed for 2 hr on the free-swimming second-stage larvae and maintained at 30 C for 6 days. Each loach captured in Tsu City was placed in a bottle containing a shallow layer of water and allowed to feed for 5 hr on various numbers of copepods infected with early third-stage larvae. These loaches were maintained at 27 C until examined. The muscles and viscera were pressed between 2 thick glass plates and searched for nematodes with the aid of a dissecting microscope.

## RESULTS

### Investigation of *G. nipponicum* in the field

Only 7 larval gnathostomes, 2 from viscera and 5 from muscles, were recovered from 3,098 loaches (Table I; Fig. 2). One larva was recovered from 311 loaches captured in August 1986, 5 from 551 loaches (single nematode, each) in September 1986, and 1 from 143 loaches in April 1987. No larvae were found from 2,093 loaches captured in the winter season.

The head bulb of the larvae had 3 transverse rows of hooklets with oblong bases, and the hooklets in each row increased in number posteriorly. The whole body was encircled by more than 200 transverse rows of minute cuticular spines. The club-shaped esophagus was slightly yellowish and the intestine was brownish. Body length and width, hooklets and their bases on the head bulb, and cuticular spines were greater in

from viscera. Bar = 20  $\mu\text{m}$ . 4. Larva from muscle. Note more developed hooklets, their bases, and cuticular spines as compared to those of larva from viscera. Bar = 20  $\mu\text{m}$ . 5. Larva encapsulated in the muscle of experimentally infected loach under laboratory conditions (day 100, 350  $\times$  350  $\mu\text{m}$ ).

TABLE II. *Experimental infection of loaches with early third-stage larvae of G. nipponicum in Cyclops vicinus and Thermocyclops hyalinus.*

Loach no.	No. of copepods ingested	Total no. of larvae	Days postinfection	No. of larvae recovered	% Recovery	Body length (mm)	Encapsulation	
							+	-
1	50	50	9	8	16.0	0.65-1.00	0	8
2	50	50	15	2	4.0	1.00-1.10	0	2
3	50	50	20	3	6.0	0.95-1.30	0	3
4	10	17	32	2	11.8	0.95-1.36	0	2
5	50	110	60	12	10.9	1.10-1.40	2	10
6	50	110	100	20	18.2	1.10-1.88	20	0

larvae from muscles than those from viscera (Figs. 3, 4).

Six weasels from the same geographical area were examined for adult worms. At autopsy, 80 adult *G. nipponicum* were recovered from esophageal tumors of 4 weasels (37, 18, 13, and 12, each). Two weasels were determined positive by recovery of eggs (avg.  $73.7 \times 42.0 \mu\text{m}$ ) of the same species from feces.

#### Experimental infection of loaches

Attempts to infect 25 loaches with early third-stage larvae in copepods were all successful. We recovered 370 larvae and all were from muscle except 2 from the viscera. The highest rate of recovery was 33.0% and the lowest was 3% (avg.  $13.8 \pm 8.3\%$  SD). Table II shows 6 representative results of 25 loaches at 9-100 days postinfection. Range in body length of larvae in copepods was between 450 and 650  $\mu\text{m}$  ( $n = 24$ ,  $\bar{x} = 520.2 \mu\text{m}$ ), and larvae ingested by loaches grew rapidly during the early period of infection. Means and ranges of measurements of 10 larvae are shown to compare with those of larvae from naturally infected loaches (Table I, bottom). However, there was no morphological difference between them in particular. The larvae lay in coils in muscle on day 9 postinfection, but they were not encapsulated. Two of 12 larvae were found encapsulated in the muscle on day 60, but the cyst wall was very thin. Encapsulated larvae in fully developed cysts were recovered on day 100 postinfection (Fig. 5).

#### DISCUSSION

Larval gnathostomes of 3 native species, *G. spinigerum*, *G. doloresi*, and *G. nipponicum*, have been found in cold-blooded vertebrates by Yoh (1946), Miyazaki and Ishii (1952), and Koga and Ishii (1981), respectively, in Japan. Moreover, larval *G. hispidum* was found in loaches im-

ported to Japan from southeast Asia (Akahane et al., 1982).

Morphological features of larval *G. nipponicum* are quite different from those of the other *Gnathostoma* species in Japan. Detailed morphological differences of advanced third-stage larvae were described between *G. spinigerum* and *G. doloresi* (Miyazaki and Ishii, 1952), *G. nipponicum* (Koga and Ishii, 1981), and *G. hispidum* (Kondo et al., 1984). The most striking differences between *G. nipponicum* and the other 3 species were the number of transverse rows of hooklets on the head bulb. The head bulb of *G. nipponicum* has 3 transverse rows of hooklets and the other 3 species have 4 rows. The hooklets with oblong bases increase in number posteriorly in *G. nipponicum*, and the whole body is covered with more than 200 transverse rows of cuticular spines. The larva has a slender esophagus and is almost colorless except for the brownish intestine. These morphological features were seen in the early third-stage larvae of *G. nipponicum* in copepods (Miyazaki, 1952a; Arita, 1953). The morphological features of larvae recovered from viscera and muscles of loaches captured in the rice field in our study were almost identical with those of early third-stage larvae in copepods and advanced third-stage larvae from snakes, except for the yellowish esophagus.

Morphological differences in cross sections of advanced third-stage larval *G. spinigerum*, *G. hispidum*, and *G. doloresi* indicate only minor differences (Akahane et al., 1986), i.e., the number of nuclei in the cylindrical epithelial cells of the mesentery were 3-7 nuclei per cell in *G. spinigerum*, mainly 2 nuclei in *G. doloresi*, and usually 1 nucleus in *G. hispidum*. The number of nuclei per cell in the cross section in our specimen was not similar to any of those species, i.e., our specimen had 1 cell showing no nucleus, 12 cells with 1 nucleus, 6 cells with 2 nuclei, 1 cell showing 3 nuclei, and 1 cell with 4 nuclei. More-



over, there were no other larval species recovered from the loaches in our study area other than *G. nipponicum*. These facts suggest that the cross section of the larval *Gnathostoma* from the patient noted in case 1 above was probably *G. nipponicum*. Unfortunately, there is no report of cross sections of advanced third-stage *G. nipponicum* from humans to compare. We are currently attempting to infect laboratory rats in order to obtain cross sections of advanced third-stage larvae so that comparisons can be made between them and the cross sections from humans.

Data and literature comparisons presented above indicate that the 7 larvae we found were *G. nipponicum*, and that loach is a second intermediate host in the natural life cycle. Loach, *M. anguillicaudatus*, is a second intermediate host of *G. spinigerum* (Miyazaki, 1952b). It was uncertain, however, whether larvae had encapsulated in the loaches we collected because a digestion method was employed for larval recovery. Nevertheless, we experimentally infected loaches and recovered fully encapsulated larvae on day 100 postinfection.

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# HELMINTHS OF THE FLORIDA MANATEE, *TRICHECHUS MANATUS LATIROSTRIS*, WITH A DISCUSSION AND SUMMARY OF THE PARASITES OF SIRENIANS

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**ABSTRACT:** We examined 215 Florida manatees (*Trichechus manatus latirostris*) at necropsy to determine the helminth fauna. Six species were identified: *Heterocheilus tunicatus* (Nematoda: Ascaridoidea); *Anoplocephala* sp. (Cestoda: Cyclophyllidae); and 4 species of trematodes, *Cochleotrema cochleotrema* (Digenea: Opisthotrematidae), *Chiorchis fabaceus* (Digenea: Paramphistomatidae), *Nudacotyle undicola* (Digenea: Nudacotylidae), and *Moniligerum blairi* (Digenea: Opisthotrematidae). Seventy-three percent of the manatees examined were infected with at least 1 species of helminth. The mean number of species of helminths per infected manatee was 1.9 with a range of 1-4. Fifty-nine manatees were helminth-free; 30 of these were calves.

No associations were found between the intensity of helminth infections and host sex, age class, season, and geographic location of recovery, or cause of death. Differences in parasite prevalence between age classes were highly significant for *Chiorchis*, *Cochleotrema*, and *Heterocheilus*, due to a low number of infected calves. A higher prevalence of *Cochleotrema* was found in manatees recovered from eastern Florida, and *Heterocheilus* was evident in significantly more manatees from western and southern Florida. Comparisons in the parasite fauna are made among Florida manatees and other sirenian populations, and a brief review of sirenian parasites is included.

The order Sirenia includes 4 extant species of aquatic, tropical, herbivorous mammals: 3 manatees (Trichechidae) and the dugong (Dugongidae) (Fig. 1). The Amazonian manatee (*Trichechus inunguis* Natterer) is confined to freshwater areas of the Amazon River Basin, whereas the West African manatee (*Trichechus senegalensis* Link) and the West Indian manatee (*Trichechus manatus* Linnaeus) range in both freshwater and saline habitats. Two subspecies of *T. manatus* are recognized: *T. manatus latirostris* in Florida, and *T. manatus manatus* in other parts of the range. The exclusively marine dugongs (*Dugong dugon* Lacepede (Muller)) inhabit warm coastal areas of the Pacific and Indian Oceans. All sirenians are endangered or threatened throughout their range (Thornback and Jenkins, 1982).

The parasite fauna of sirenians is poorly known. Our objectives were to determine the helminth fauna of *T. manatus latirostris* in Florida, report the prevalence and intensity of each species, and summarize the known parasite fauna of the Sirenia. Three helminths (*Chiorchis fabaceus*, *Cochleotrema cochleotrema*, and *Heterocheilus tun-*

*icatus*) in Florida manatees were previously documented (Hutton, 1964; Radhakrishnan and Bradley, 1970; Forrester et al., 1975, 1980; Blair, 1981b), with 2 additional unidentified trematodes also noted (Forrester et al., 1980; Reynolds, 1980). Forrester et al. (1975) reported 2,659 *Chiorchis* specimens from 1 manatee and quantitative data also were collected for 4 helminths from 48 Florida manatees (Forrester et al., 1980). Boever et al. (1977) reported an estimate of 6,000 *Chiorchis* individuals from a single *T. inunguis*, and Marsh et al. (1977) reported a 96.8% prevalence of the nematode *Paradujardinia halicoris* in 31 dugong carcasses. We know of no other studies that provide quantitative details of the helminths of sirenians.

## MATERIALS AND METHODS

Helminths were collected from dead manatees recovered throughout Florida from October 1974 through October 1982. We recovered 594 dead manatees during the study, and data on the prevalence of 5 helminths were determined from 215 of these. Prevalence was recorded for each species when host organs were intact. Stages of decomposition varied from fresh to badly decomposed (as defined by Bonde et al., 1983); poor carcass condition sometimes precluded examination of all organ systems, therefore varying the number of manatees examined for each helminth. Data on the intensity of 4 helminths were obtained from 42 of the 215 manatees. Terminology used to describe parasite infections follows Margolis et al. (1982).

The entire gastrointestinal (GI) tract was opened at necropsy and examined for *Heterocheilus* and *Chior-*

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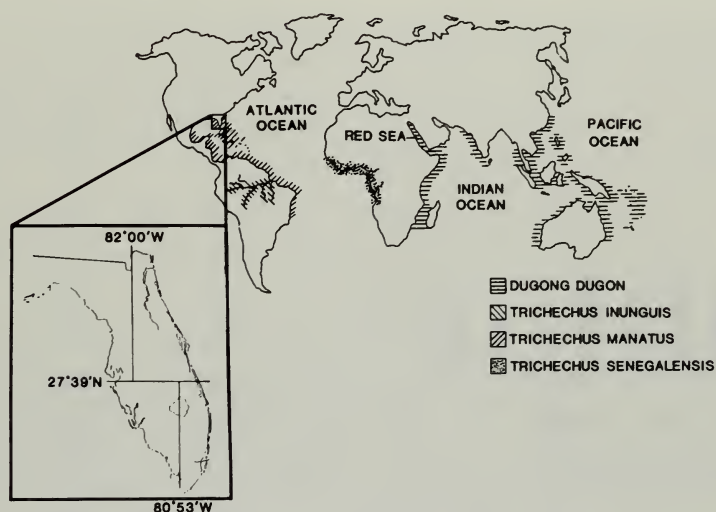


FIGURE 1. Distribution of living sirenians in the world, with inset of peninsular Florida showing division of the state into quadrants used for analyses by recovery locations.

*chis*. Samples of digesta were washed through a 100-mesh (0.15-mm) sieve and examined under a dissecting microscope to validate the presence of *Nudacotyle* and to search for other species. If condition allowed, mucosal surfaces also were examined. Sections of suspect tissue were examined microscopically or histologically. The nares and bronchi were opened and irrigated to locate *Cochleotrema*. Heart, liver, gall bladder, bile duct, pancreas, kidneys, urinary bladder, reproductive organs, eustachian tubes, and muscle also were examined.

Analyses of intensity were conducted only when the species was collected from all organs that were known sites of infection. To obtain counts of all intestinal helminths, segments of the GI tract were isolated and often were frozen prior to examination. Contents were washed through sieves to recover helminths for total counts. Counts of *Chiorchis* were recorded for the caecum and 5 equivalent subdivisions of the colon of 12 manatees, and in 13 manatees total counts of *Nudacotyle* were made from 10 equal subdivisions of the small intestine. Specimens of *Cochleotrema* were washed from the opened nares and bronchi into a screen and counted.

Carcasses were assigned to 1 of 3 age classes based on body size (calves  $\leq 175$  cm, juveniles  $> 175$  cm and  $\leq 275$  cm, and adults  $> 275$  cm) (O'Shea et al., 1985). Carcass recovery dates were categorized by season: winter (Dec–Feb), spring (Mar–May), summer (Jun–Aug), and fall (Sep–Nov). Recovery locations were categorized as east and west coasts of Florida, and as northern and southern areas of the state, divided by a line at 27°39'N latitude (Fig. 1). One of 6 cause-of-death categories was assigned at necropsy (Bonde et al., 1983).

Nonparametric statistical tests were utilized for all analyses. Contingency tables were compiled and compared to the expected frequencies to determine the prevalence of each species and to test for significant associations between prevalence and the host factors.

Data on intensity were tested for with host factors using the Mann-Whitney *U*-test and the Kruskal-Wallis test (Sokal and Rohlf, 1981).

Representative specimens of *Heterocheilus tunicatus* (No. 78641), *Cochleotrema cochleotrema* (No. 78642), *Chiorchis fabaceus* (No. 78643), *Anoplocephala* sp. (No. 78749), *Moniligerum blairi* (No. 79800), and *Nudacotyle undicola* (No. 79801) have been deposited in the U.S. National Parasite Collection (Beltsville, Maryland 02705).

## RESULTS

Six species of helminths were identified. One nematode, *Heterocheilus tunicatus* Diesing, and 2 trematodes, *Chiorchis fabaceus* (Diesing) Fischöder and *Nudacotyle undicola* Dailey, Vogelbein, and Forrester, were found commonly in the lumen of the GI tract. Another trematode, *Moniligerum blairi* Dailey, Vogelbein, and Forrester, was found encysted in the mucosa and submucosa of the small intestine of 7 manatees (Reynolds, 1980; Dailey et al., 1988). *Cochleotrema cochleotrema* Travassos and Vogelsang infected the lungs and nares. The cestode *Anoplocephala* sp. was collected from a manatee recovered from southeastern Florida. This single specimen was found in the small intestine of only 1 manatee examined and is not included in subsequent analyses. The total number of manatees examined for each species of helminth, overall prevalence, and prevalences within the selected categories are listed in Table 1.

Concurrent infections of more than 4 species were not found. Five manatees carried simul-



TABLE I. Prevalences of 5 species of helminths recovered from Florida manatees.

	Helminth*				
	C.f.	C.c.	N.u.	M.b.	H.t.
Overall % prevalence	66 (203)†	38 (146)*	18 (39)	90 (31)	39 (185)
Sex					
Male	72 (103)	39 (72)	20 (20)	89 (18)	42 (95)
Female	59 (100)	38 (74)	16 (19)	92 (13)	36 (90)
Age class					
Calf	8 (39)	3 (35)	3 (7)	67 (6)	3 (36)
Juvenile	79 (82)	55 (55)	18 (22)	95 (19)	47 (77)
Adult	79 (82)	45 (56)	20 (10)	100 (6)	49 (72)
Season					
Winter	60 (85)	44 (48)	25 (20)	92 (25)	36 (77)
Spring	69 (65)	39 (52)	37 (9)	75 (4)	37 (60)
Summer	65 (31)	29 (31)	17 (6)	100 (1)	39 (28)
Fall	77 (22)	15 (15)	0 (4)	100 (1)	55 (20)
Location					
East	67 (137)	43 (109)	18 (28)	87 (23)	33 (126)
West	62 (66)	24 (37)	18 (11)	100 (8)	53 (59)
North	62 (134)	37 (123)	33 (25)	77 (13)	8 (131)
South	72 (69)	43 (23)	36 (14)	100 (18)	54 (54)
Death category					
Human-related	82 (74)	47 (53)	17 (12)	89 (9)	44 (63)
Natural	68 (41)	54 (24)	27 (11)	92 (12)	46 (37)
Dependent calves	8 (26)	4 (27)	20 (5)	0 (1)	4 (24)
Undetermined	68 (62)	40 (42)	9 (11)	100 (9)	43 (61)

\* C.f. = *Chiorchis fabaceus*, C.c. = *Cochleotrema cochleotrema*, N.u. = *Nudacotyle undicola*, M.b. = *Moniligerum blairi*, H.t. = *Heterocheilus tunicatus*.

† Numbers in parentheses are the number of manatees examined in each category.

taneous infections of 4 helminths; 37 were infected with 3 species; 53 manatees were infected with 2 species, and 1 species of helminth was found in 61 of the manatees. Thirty of the 59 manatees that were not infected with parasites were calves.

Differences in prevalence between host sexes or recovery seasons were not significant for any species of helminth (Table I). Differences in prevalence between age classes were significant ( $P = 0.0001$ ) for *Chiorchis*, *Cochleotrema*, and *Heterocheilus*, due to the low number of infected calves. Sample sizes were insufficient for statistical analyses between age classes for *Nudacotyle* and *Moniligerum*; however, all age classes were infected with these trematodes.

No association between cause of death and prevalence was found for any helminth species. Documentation of parasites contributing to manatee deaths or disease was rare. However, 2 deaths were coincident with *Cochleotrema* infections of high intensity. An adult male recovered in January 1979 had at least 250 nasal flukes (*Cochleotrema*) present in the nares and mouth, resulting in severe, chronic rhinitis and pulmonary edema. A juvenile female recovered

in January 1981 harbored a minimum of 490 individuals of *Cochleotrema* in the nares, trachea, bronchi, and bronchioles, resulting in severe, chronic, ulcerative epiglottitis and pneumonia. Death was attributed to verminous pneumonia (Buerfelt et al., 1984). Additionally, a young calf recovered in February 1977 was found with severe hemorrhagic enteritis, coincident with the highest recorded count of *Nudacotyle* (132,110).

*Heterocheilus* was found in more manatees from Florida's west coast ( $P = 0.01$ ) and from southern Florida ( $P = 0.009$ ) than from other areas. *Cochleotrema* occurred more often in manatees from eastern Florida ( $P = 0.038$ ). There was no difference due to latitude for prevalence of *Cochleotrema*. No association between recovery location and prevalence was found for *Chiorchis*. Sample sizes of recovery locations were too small to test for associations for the remaining species (Table I).

Data on intensity were available for 4 helminths from 42 manatees. However, the total number available for analyses of each species varied depending on organ condition (Table II). Although no association existed between the in-

TABLE II. Intensities of 4 helminths from Florida manatees.

Helminth	Sample size	Intensity		
		Mean	Median	Range
<i>Chiorchis</i>	29	2,731	1,145	15–24,976
<i>Cochleotrema</i>	19	54	27	2–250
<i>Nudacotyle</i>	4	44,223	22,306	169–132,110
<i>Heterocheilus</i>	19	228	51	2–1,693

tensity of any of the 4 helminths and host sex, age class, recovery season or location, or cause of death category, one finding was notable: The larger, and therefore older, manatees had the greatest number of individual parasites, probably due to the larger available habitat within the host, as well as to an increased opportunity of exposure due to age (with the exception of the high count of more than 132,000 *Nudacotyle*, recorded from a calf).

The distribution of the 4 species of helminths within the host revealed site preferences (Table III). The small intestine harbored 99.8% of all *Nudacotyle* individuals counted in 4 manatees. However, within subdivisions of this organ, the distribution of nearly 2 million specimens of *Nudacotyle* plotted for 13 carcasses was fairly even, with only a slightly higher percentage at the distal end (Fig. 2). Most (85.4%) *Chiorchis* specimens were recovered from the colon, and the remainder concentrated primarily in the caecum. The distribution of nearly 16,000 *Chiorchis* individuals in the large intestine of 12 manatees was concentrated in the caecum and proximal colon (Fig. 3). *Heterocheilus* counts were highest (90.6% of the total) in the stomach. Most (89.9%) specimens of *Cochleotrema* were recovered from the nares, with most of the remainder found in the primary bronchi. The distribution of helminths in a single host was consistent with the overall distribution of these species in all manatees included in the analyses.

### DISCUSSION

Five of the helminth species reported from Florida manatees were recovered throughout the state. *Heterocheilus* and *Cochleotrema* apparently were more prevalent in some locales of Florida, but it is unknown if this reflected variation in the distribution or abundance of the definitive or intermediate hosts, habitat variables such as salinity or temperature, or other factors. Knowledge of the life cycles of these parasites

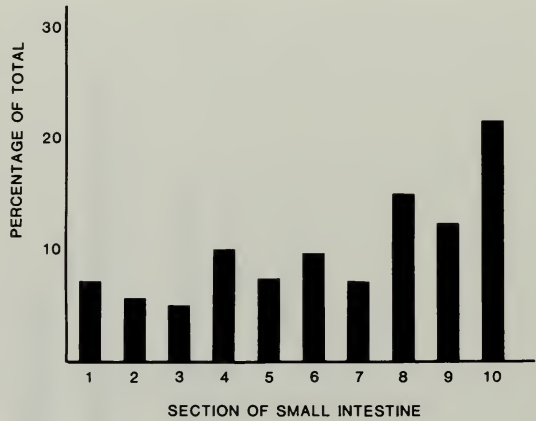


FIGURE 2. Distribution of 1,836,579 *Nudacotyle* individuals in 10 equal sections of the small intestine of 13 manatees.

would aid in understanding their distribution, but such information is completely unknown.

Lack of significant seasonal variation in prevalence or intensity may be explained by a long survival time of a parasite within the host, an abundant supply of resistant larvae or intermediate hosts, or a high fecundity of the parasite (Rohde, 1982, 1984). Differences in parasite prevalence between calves and older animals were expected. Calves have less opportunity to ingest infective stages because they consume less vegetation than larger animals, and have been alive for less time to allow for exposure or develop-

TABLE III. Distribution within the host of 4 species of helminths recovered from 42 Florida manatees.

Helminths (sample size)	Total no. specimens	Percentage of total
<i>Chiorchis</i> (29)		
Colon	67,669	85.4
Caecum	10,831	13.7
Small intestine	564	0.7
Stomach	133	0.2
<i>Cochleotrema</i> (19)		
Nares	930	90.0
Lungs	83	8.0
Stomach	21	2.0
<i>Nudacotyle</i> (4)		
Small intestine	176,608	99.8
Other (duodenum, caecum, colon)	283	0.2
<i>Heterocheilus</i> (19)		
Stomach	3,924	90.6
Duodenum	189	4.4
Small intestine	151	3.5
Colon	56	1.3
Caecum	11	0.2



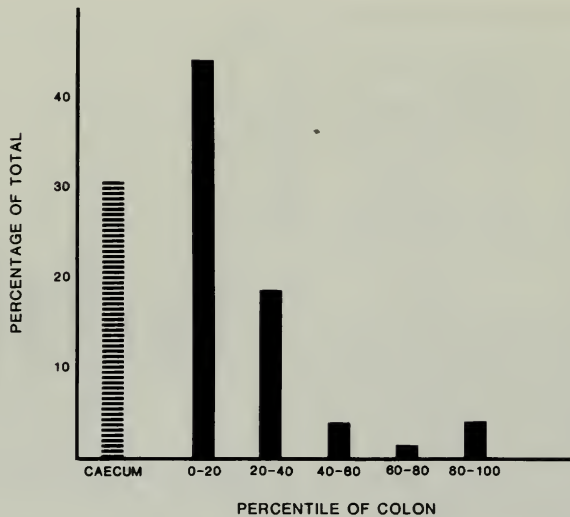


FIGURE 3. Distribution of 15,756 *Chiorchis fabaceus* individuals in the large intestine of 12 manatees.

ment of the helminths. No significant difference in the infections of *Chiorchis*, *Cochleotrema*, and *Heterocheilus* between juveniles and adults may indicate a lack of immunity to these helminths with increasing age.

The prevalence and intensity reported for *Cochleotrema* may be underestimated because these trematodes are easily lost from the nares of carcasses, while they remain in the water after death and during transport prior to necropsy. *Moniligerum* also may be more common than the 18% prevalence reported here; the small mucosal cysts are difficult to discern except in fresh carcasses, which are seldom available. The infection of 1 specimen of *Anoplocephala* sp. in a single manatee likely was accidental and of equine origin. The intermediate hosts of *Anoplocephala* are free-living mites; shore-browsing, common by manatees, might have permitted its acquisition.

Although the West Indian manatee has a wide distribution (Fig. 1), the parasite fauna has been documented most thoroughly for *T. manatus* in Florida (Table IV). *Chiorchis* has been reported in this host in Mexico (Sokoloff and Caballero C., 1932; Dollfus, 1955; Lluch, 1965; Bravo-Hollis and Caballero Deloya, 1973), Brazil (Stedman, 1889), and Guyana (Chapman, 1875), and *Cochleotrema* also is known from manatees in Guyana (Dollfus, 1955) and elsewhere in the Caribbean (Travassos and Vogelsang, 1931; Dollfus, 1955). Khalil and Vogelsang (1932) and Sprent (1980) have examined *Heterocheilus*

specimens from *T. manatus* recovered in the Caribbean. These 3 species may be common in West Indian manatees throughout their range. Prevalence data on parasites are not available for *T. inunguis*, *T. senegalensis*, or *T. manatus* outside of Florida.

The southeastern United States is the northern limit of *T. manatus* and the parasites identified in Florida manatees may be minimal in both diversity of species present and the prevalence and intensity of the infections compared to manatees recovered nearer the center of the host's range. Populations of manatees in Florida are well enough isolated to warrant subspecific designation (Hatt, 1934; Domning and Hayek, 1986), and geographic barriers between the subspecies may have resulted in at least a limited division in the parasite fauna as well. The more seasonal climate of Florida also may influence parasite ecology and life cycles. A comparison of the helminth fauna of Florida manatees to that of *T. manatus manatus* south of Florida will be required to determine the full range of helminth species utilizing this host.

All known parasites of sirenians are listed by host species in Table IV. The greater diversity of helminths recorded from dugongs may be due to a greater research effort to document the parasite fauna of this sirenian, or due to their unique environment. The warm, marine habitat of the dugong and the generally richer marine fauna of the tropical Pacific offer a larger selection of intermediate hosts (Delyamure, 1955; Rohde,

TABLE IV. *A summary of the parasites and cirripeds reported from sirenians.*

		Site	Source
<i>Trichechus manatus latirostris</i>			
TREMATODA			
Paramphistomidae	<i>Chiorchis fabaceus</i>	Lumen of caecum and colon	Fischeoder, 1901, 1902; Gudernatsch, 1908; Stunkard, 1929; Price, 1932; Canavan, 1934; Travassos, 1934; Dollfus, 1955; Hutton, 1964; Radhakrishnan and Bradley, 1970; Forrester et al., 1975, 1980; Bonde et al., 1983; Buergelt et al., 1984
Opisthotrematidae	<i>Cochleotrema cochleotrema</i>	Nares, lungs	Price, 1932; Dollfus, 1955; Forrester et al., 1980; Blair, 1981b; Bonde et al., 1983; Buergelt et al., 1984
	<i>Moniligerum blairi</i>	Encapsulated in wall of small intestine	Reynolds, 1980; Dailey et al., 1988
Nudacotylidae	<i>Nudacotyle undicola</i>	Lumen of small intestine	Forrester et al., 1980; Dailey et al., 1988
NEMATODA			
Ascaridae	<i>Heterocheilus tunica-tus</i>	Lumen and mucosa of stomach, and small intestine	Radhakrishnan and Bradley, 1970; Forrester et al., 1980; Sprent, 1980, 1983; Bonde et al., 1983
CESTODA			
Anoplocephalidae	<i>Anoplocephala</i> sp.	Lumen of small intestine	Present paper
PROTOZOA			
Apicomplexa	<i>Toxoplasma gondii</i>	Brain	Buergelt and Bonde, 1983
CRUSTACEA			
Copepoda	<i>Harpacticus pulex</i>	Skin	Humes, 1964
Cirripedia	<i>Chelonibia manati</i> sp. aff.	Skin	Spivey, pers. comm.
<i>Trichechus manatus manatus</i>			
TREMATODA			
Paramphistomidae	<i>Chiorchis fabaceus</i>	Lumen of caecum and colon	Chapman, 1875; Stedman, 1889; Sokoloff and Caballero C., 1932; Lluch, 1965; Bravo-Hollis and Caballero Deloya, 1973
Opisthotrematidae	<i>Cochleotrema cochleotrema</i>	Nares, lungs	Travassos and Vogelsang, 1931; Price, 1932; Dollfus, 1955
NEMATODA			
Ascaridae	<i>Heterocheilus tunica-tus</i>	Lumen and mucosa of stomach, and small intestine	Khalil and Vogelsang, 1932; Sprent, 1980
<i>Trichechus inunguis</i>			
TREMATODA			
Paramphistomidae	<i>Chiorchis fabaceus</i>	Lumen of caecum and colon	Diesing, 1838, 1839; Fischeoder, 1901, 1902; Stunkard, 1929; Price, 1932; Travassos 1934; Baylis, 1936; Dollfus, 1955; Travassos et al., 1969; Boever et al., 1977
NEMATODA			
Ascaridae	<i>Heterocheilus tunica-tus</i>	Lumen and mucosa of stomach, and small intestine	Diesing, 1839; Dollfus, 1955; Sprent, 1980
PROTOZOA			
Apicomplexa	<i>Eimeria trichechi</i>	Oocysts in feces	Lainson et al., 1983
<i>Trichechus senegalensis</i>			
TREMATODA			
Paramphistomidae	<i>Chiorchis fabaceus</i>	Lumen of caecum and colon	Fischeoder, 1901; Derscheid, 1926; Stunkard, 1929; Price, 1932; Travassos, 1934; Baylis, 1936; Dollfus, 1955
NEMATODA			
Ascaridae	<i>Heterocheilus domningi</i>	Unknown	Sprent, 1983
CRUSTACEA			
Cirripedia	<i>Chelonibia manati</i>	Skin	Gruvel, 1903; Pilsbry, 1916; Stubbings, 1965
	<i>Platylepas hexastylus</i>	Skin	Stubbings, 1965

1982). However, the diversity of parasite species in dugongs also may be due to their longer evolutionary history in a marine environment. The sirenians are thought to have arisen in a marine habitat, the manatees having entered freshwater

areas relatively recently in the Late Miocene (Domning, 1982). Hosts that have inhabited an area for the longest time are likely to have the greatest diversity of parasites (Rohde, 1982), and dugongs are from an older group that has re-



TABLE IV. *Continued.*

		Site	Source
<i>Dugong dugon</i>			
TREMATODA			
Paramphistomidae	<i>Solenorchis baeri</i> *	Caecum	Hilmy, 1949; Dollfus, 1955; Gohar, 1957; Nair et al., 1975;
	<i>S. gohari</i> *	Caecum	Blair, 1980, 1981a; Sey, 1980
	<i>S. naguibmah-fouzi</i> *	Caecum	
	<i>S. travassosi</i> *	Caecum	
	<i>Indosolenorchis hirudinaceus</i> *	Caecum and proximal colon	Crusz, 1951; Crusz and Fernand, 1954; Dollfus, 1955; Nair et al., 1975; Allen et al., 1976; Kamegai, 1979; Blair, 1980, 1981a; Sey, 1980
Opisthotrematidae	<i>Zygocotyle</i> sp.*	Caecum	Dollfus, 1950, 1955
	<i>Opisthotrema dujonis</i>	Eustachian tubes, middle ear, esophagus	Fischer, 1884; Price, 1932; Dollfus, 1955; Nair et al., 1975; Allen et al., 1976; Blair, 1981a, 1981b
	<i>Opisthotrema australe</i>	Eustachian tubes, middle ear	Blair, 1981b
	<i>Pulmonicola pulmonalis</i> †	Lungs	Von Linstow, 1905; Price, 1932; Nair et al., 1975; Blair, 1981b
	<i>Cochleotrema indicum</i>	Nares, lungs	Sharma and Gupta, 1971; Kamegai, 1979; Sey, 1980; Blair, 1981a, 1981b
	<i>Lankatrema mananarensis</i>	Wall of stomach, duodenum, and small intestine	Crusz and Fernand, 1954; Nair et al., 1975; Marsh et al., 1977; Blair, 1981a, 1981b
	<i>L. minutum</i>	Wall of cardiac gland	Blair, 1981b
	<i>L. microcotyle</i>	Wall of ileum	Blair, 1981b
	<i>L. macrocotyle</i>	Wall of ileum	Blair, 1981b
	<i>Lankatrematoides gardneri</i>	Pancreatic ducts	Blair, 1981b
	<i>Folitrema jecoris</i>	Gall bladder and bile ducts within liver	Blair, 1981b
Labicolidae	<i>Labicola elongata</i>	Abscesses in upper lip	Blair, 1979, 1981a
Rhabdiopoeidae	<i>Rhabdiopoeus taylori</i>	Lumen of intestines	Johnston, 1913; Price, 1932; Dollfus, 1955; Gohar, 1957; Nair et al., 1975; Kamegai, 1979; Blair, 1981a, 1981b
	<i>Taprobanella bicaudata</i>	Stomach, duodenum, pyloric caeca	Crusz and Fernand, 1954; Nair et al., 1975; Kamegai, 1979; Blair, 1981a, 1981b
	<i>Haerator caperatus</i>	Lumen of ileum	Blair, 1981b
	<i>Faredifex clavata</i>	Large abscesses in wall of ileum	Blair, 1981b
	Eggs of unknown species	Ovaries, myometrial blood vessels	Marsh et al., 1984
NEMATODA			
Ascaridae	<i>Paradujardinia hali-coris</i>	Lumen of stomach, cardiac gland	Owen, 1838; Baird, 1859; Diesing, 1861; Von Linstow, 1905; Travassos, 1933; Johnston and Mawson, 1941; Crusz and Fernand, 1954; Dollfus, 1955; Gohar, 1957; Nair et al., 1975; Jueco, 1977; Marsh et al., 1977; Sprent, 1980; Blair, 1981a
CRUSTACEA			
Cirripedia	<i>Platylepas hexastylus</i>	Skin	Pilsbry, 1916; Marlow, 1962

\* Dollfus (1955) proposes that these names apply to only 1 species of *Zygocotyle*; Sey (1980) considers these species synonymous with *S. travassosi*. Blair (1980) considers *Zygocotyle* sp. a synonym of *I. hirudinaceus*, but supports retention of *Solenorchis* as a distinct genus.

† Blair (1981b) considers *P. pulmonalis* a species inquirenda, possibly synonymous with *C. indicum*.

mained in the marine habitat of ancestral sirenians. The euryhaline habits of the Florida manatees also may account for the paucity of parasites reported from this host (Hartman, 1979).

Life cycles have not been determined for any of the parasites of sirenians. Most species in the cosmopolitan family Ascaridae have direct life cycles (Levine, 1968). Sprent (1980) proposed the subfamily Heterocheilinae to include the species from freshwater turtles and fish, crocodilians, and sirenians, including the species of

*Heterocheilus* in manatees and *Paradujardinia* in dugongs. He hypothesized that these species use a crustacean as an intermediate host. Sirenians conceivably could become infected in either manner: Coprophagy has been documented (Hartman, 1979) and the eggs could be ingested with the feces or while feeding on contaminated vegetation, as suggested by Jueco (1977). The host also could be infected when crustaceans are consumed incidentally while feeding on vegetation. If the life cycle of the ascarids of sirenians

is indirect, then the intermediate host selection for these species may be broad, perhaps with both marine and freshwater crustaceans capable of maintaining the larval stages.

*Chiorchis* is a paramphistome common to all manatees, including the purely freshwater *T. inunguis*. Paramphistome cercaria of mammals encyst on vegetation (Stunkard, 1929), and freshwater snails act as intermediate hosts (Yamaguti, 1971). The intermediate host(s) may be a freshwater snail, with transmission occurring when manatees ingest metacercariae while feeding. However, paramphistomes occur in dugongs in the Red Sea (Blair, 1981a), and it is therefore possible that the life cycle also could be perpetuated with a marine mollusc serving as the intermediate host.

The genus *Cochleotrema* is found in the nares and lungs of both *T. manatus* and dugongs. Because the former ranges into saltwater habitats and the latter is exclusively marine, it is likely that the larvae of these trematodes use a marine invertebrate in their development.

A complete investigation of the parasites of *T. manatus* nearer the center of its distribution, and of *T. inunguis* and *T. senegalensis*, undoubtedly would add more parasites to the list of known species in manatees. Continued research efforts are required to enhance our understanding of these marine mammals and their parasite fauna.

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## DIGENETIC TREMATODES OF MARINE FISHES OF OKINAWA, JAPAN

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**ABSTRACT:** Between May and September 1985, 348 fishes representing 50 families, 107 genera, and 152 species from the coastal waters of Okinawa were examined for digenetic flukes. Ten families (Lepocreadiidae, Opisthophlebetidae, Gyliauchenidae, Fellodistomidae, Acanthocolpidae, Opecoelidae, Bucephalidae, Cryptogonimidae, Syncoeliidae, and Hemiuridae), representing 29 genera and 34 species of digenetic flukes were recorded. Seven new geographic locality records and 25 new host records were established. Possibly 2 new species, one being a species of the genus *Metadena* from *Meiacanthus grammistes* and the other a species of the genus *Mesolecitha* from *Plectorhynchus chaetodontoides*, were detected. Most infections were of a single species, and although prevalence and intensity were low, host specificity was high. Only 3 of the 34 species identified transgressed family bounds in their definitive hosts.

Although contributions to our knowledge of the digenetic flukes of marine fishes of the Japanese Archipelago have been made by numerous workers, only 1 comprehensive report, namely, that of Yamaguti (1942) deals with the digeneans of marine fishes of Okinawa. Because of the need for additional information concerning the digenetic flukes of marine fishes of Okinawa, the present investigation was undertaken to examine a large number of fishes over a 5-mo period. In some instances, flukes were either immature or too few to render identification possible. In addition, there are unsatisfactory representatives of possibly 2 new species; the material, however, is inadequate to serve as the basis for describing them as new. One is a species of the genus *Metadena* from *Meiacanthus grammistes* (Valenciennes) and the other is a species of the genus *Mesolecitha* from *Plectorhynchus chaetodontoides* (Lacepède).

### MATERIALS AND METHODS

Between May and September 1985, 348 marine fishes, representing 49 families of bony fishes and 1 family of cartilaginous fishes from the coastal waters of Okinawa, were examined for the presence of digenetic flukes. Fishes were collected by means of a variable mesh gill net, seine, traps, spearfishing, and hook and line supplemented by quinaldine, and rotenone to obtain species inhabiting shallow water, placed in plastic bags containing seawater, and held in styrofoam containers for transport to the laboratory where they were refrigerated and usually necropsied shortly thereafter.

Flukes were washed in 0.7% saline, fixed in warm AFA under slight coverslip pressure, stored in 70% ethanol, stained with either Harris' hematoxylin or

Grenacher's alcoholic borax carmine, and prepared as whole mounts in Canada balsam. Voucher specimens of most species have been deposited in the National Parasite Collection, USDA, Beltsville, Maryland, under the accession numbers listed in Table I. Other specimens are in the author's collections.

Locality coordinates are given for each host except the whale shark, *Rhincodon typus* Smith, that was captured just southwest of Okinawa by fishermen.

### RESULTS

One or more species of digenetic flukes was recovered from 59 (17.0%) of 348 fishes. Twenty-six (52.0%) of 50 families, 35 (32.7%) of 107 genera, and 45 (29.6%) of 152 species of fishes were infected. The 34 species of flukes recovered represented 10 families (Lepocreadiidae, Opisthophlebetidae, Gyliauchenidae, Fellodistomidae, Acanthocolpidae, Opecoelidae, Bucephalidae, Cryptogonimidae, Syncoeliidae, and Hemiuridae) and 29 genera (Table I). Of the 45 species of fishes that were infected, 36 (80.0%) harbored 1 species of digenetic fluke, 7 species (15.6%) 2, and 2 others (4.4%) with 3. Negative fish are listed in Appendix I.

The intensity of infectivity by a given species ranged from 1 to 200 flukes per host. Each of 42 fishes yielded 1-5 specimens; 12, 6-12; 2, 17-20; and 3 yielded 37, 50, and 200 specimens, respectively.

For most of the fish species examined, the prevalence of infection with digenetic flukes was low. However, 42.9% of 7 "kanmonhata," the local name for *Epinephelus merra* (Bloch), were infected with *Opecoelus sphaericus* Ozaki, 1925. Twenty-seven (79.4%) of the 34 species of digenetic flukes recovered occurred in 1 host species, 4 (11.8%) in 2, 2 (5.9%) in 3, and 1 (2.9%) in 4.

Seven of the 34 trematode species recovered

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TABLE 1. Digeneic flukes of marine fishes from coastal water of Okinawa, Japan.

Hosts (no. examined/no. infected)	Parasite	Locality	No. hosts examined/no. infected/avg. no. worms per infected host	USNM Helm. Coll. No.
<b>Chondrichthyes</b>				
<b>Rhincodontidae</b>				
<i>Rhincodon typus</i> Smith (1/1)	<i>Paronatrema mantae</i> Manter, 1940		1/1/200	79988
<b>Osteichthyes</b>				
<b>Acanthuridae</b>				
<i>Acanthurus bartene</i> Lesson (1/1)	<i>Monoleithotrema kala</i> Yamaguti, 1970	26°38.03'N; 127°51.71'E	1/1/4	79957
<i>Acanthurus bleekeri</i> Günther (1/1)	<i>Proplectes xesuri</i> (Yamaguti, 1940)	26°39.71' to 39.96'N; 127°52.09' to 52.50'E	1/1/50	79958
<i>Acanthurus glaucopareus</i> Cuvier (2/2)	<i>Hysteroleclitha nahaensis</i> Yamaguti, 1942	26°39.71' to 39.96'N; 127°52.09' to 52.50'E	2/2/1	79959
<i>Acanthurus lineatus</i> (Linnaeus) (1/1)	<i>Lecithophyllum fuscum</i> Yamaguti, 1938	26°39.71' to 39.96'N; 127°52.09' to 52.50'E	1/1/1	79960
<b>Apogonidae</b>				
<i>Apogon arobianensis</i> (Hombron and Jacquinot) (4/1)	<i>Opegaster ditrematis</i> Yamaguti, 1942	26°37.95'N; 127°52.00'E	1/1/2	79887
<i>Apogon doederleini</i> Jordan and Snyder (45/2)	<i>Plagioporus (Plagioporus) apogonichthydis</i> Yamaguti, 1938	26°37.95'N; 127°52.00'E	2/1/6	79888
	<i>Helicometra (Helicometra) hypoditis</i> Yamaguti, 1934	26°37.95'N; 127°52.00'E	5/1/3	79880
<i>Cheilodipterus quinquelineatus</i> Cuvier (39/2)	<i>Helicometra (Helicometra) hypoditis</i> Yamaguti, 1934	26°37.95'N; 127°52.00'E	8/2/10	79961 79881
<b>Ballistidae</b>				
<i>Rhinecanthus aculeatus</i> (Linnaeus) (1/1)	<i>Lepocreadium clavatum</i> (Ozaki, 1932)	26°37.95'N; 127°52.00'E	1/1/12	79962
<b>Belontiidae</b>				
<i>Tylosurus crocodilus crocodilus</i> (Le Sueur) (1/1)	<i>Prosorhynchus (Skrajabinella) uniporus</i> Ozaki, 1924	26°37.95'N; 127°52.00'E	1/1/2	79738
<b>Blenniidae</b>				
<i>Meiacanthus grammistes</i> (Valenciennes) (2/1)	<i>Metadene pagrosomi</i> Yamaguti, 1938	26°37.95'N; 127°52.00'E	1/1/1	79963
<i>Plagiotremus laudandus laudandus</i> Whitley (2/1)	<i>Bucephalopsis ovatus</i> Ozaki, 1928	26°38.03'N; 127°51.71'E	1/1/6	79733
<i>Plagiotremus tapeinosoma</i> Bleeker (2/2)	<i>Bucephalopsis ovatus</i> Ozaki, 1928	26°38.03'N; 127°51.71'E	1/1/6	79734
	<i>Paragyliauchen chaetodontis</i> Yamaguti, 1935		—/—/3	79964
	<i>Bucephalopsis</i> sp.	26°37.95'N; 127°52.00'E	1/1/1	79735



TABLE I. Continued.

Hosts (no. examined/no. infected)	Parasite	Locality	No. hosts examined/no. infected/avg. no. worms per infected host	USNM Helm. Coll. No.
<b>Chaetodontidae</b>				
<i>Hemirhamphus chrysostomus</i> Cuvier (3/1)	<i>Hysterolethia</i> sp.	26°39.71' to 39.96'N; 127°52.09' to 52.50'E	2/1/8	79965
<b>Cirrhitidae</b>				
<i>Cirrhitichthys falco</i> Randall (3/1)	<i>Plagioporus (Plagioporus) apogonichthydis</i> Yamaguti, 1938	26°37.95'N; 127°52.00'E	1/1/1	79889
<b>Diodontidae</b>				
<i>Diodon holocanthus</i> Linnaeus (5/1)	<i>Opistholebes elongatus</i> Ozaki, 1937	26°37.95'N; 127°52.00'E	1/1/7	79966
<b>Ephippidae</b>				
<i>Platax pinnatus</i> Linnaeus (1/1)	<i>Paradisogaster chaetodontis okinawaensis</i> Yamaguti, 1971	26°37.95'N; 127°52.00'E	1/1/3	78985
<i>Platax teira</i> (Forsskål) (1/2)	<i>Tergesita laticollis</i> (Rudolph, 1819)	26°38.03'N; 127°51.71'E	1/2/5	79967
<b>Fistulariidae</b>				
<i>Fistularia petimba</i> Lacepède (2/2)	<i>Allolepidapedon fistulariae</i> Yamaguti, 1940	26°39.71' to 39.96'N; 127°52.09' to 52.50'E	1/1/3	79968
	<i>Opecoelus sphaericus</i> Ozaki, 1925	26°39.71' to 39.96'N; 127°52.09' to 52.50'E	1/1/2	79969
	<i>Stephanostomum fistulariae</i> (Yamaguti, 1940)		—/—/5	79970
<b>Girellidae</b>				
<i>Girella meina</i> Jordan and Starks (1/1)	<i>Opecoelus sphaericus</i> Ozaki, 1928	26°22'N; 127°05'E	1/1/2	79886
<b>Lethrinidae</b>				
<i>Lethrinus harak</i> (Forsskål) (4/2)	<i>Hamacreadium lethrini</i> Yamaguti, 1934	26°37.95'N; 127°52.00'E	2/1/3	79877
	<i>Helicometra (Helicometra) hypodytis</i> Yamaguti, 1934	26°37.95'N; 127°52.00'E	1/1/3	79879
<i>Monotaxis grandoculis</i> (Forsskål) (2/1)	<i>Pycnadenoides pagrosomi</i> Yamaguti, 1938	26°22'N; 127°05'E	1/1/3	79971
<b>Lujanidae</b>				
<i>Lujanus fuvillamma</i> (Forsskål) (3/3)	<i>Hamacreadium lethrini</i> Yamaguti, 1934	26°38.03'N; 127°51.71'E	2/2/5	79878
	<i>Stephanostomum</i> sp.	26°38.03'N; 127°51.71'E	1/1/2	79972

TABLE I. Continued.

Hosts (no. examined/no. infected)	Parasite	Locality	No. hosts examined/no. infected/avg. no. worms per infected host	USNM Helm. Coll. No.
<b>Malacanthidae</b>				
<i>Malacanthus latovittatus</i> (Lacepède) (1/1)	<i>Manteriella crassa</i> (Manter, 1947)	26°39.71' to 39.96'N; 127°52.09' to 52.50'E	1/1/3	79883
<b>Monacanthidae</b>				
<i>Cantherhines pardalis</i> (Ruppell) (2/2)	<i>Bianium</i> sp.	26°39.71' to 39.96'N; 127°52.09' to 52.50'E	2/2/4	79973
<b>Mugiloididae</b>				
<i>Paraperis cylindrica</i> (Bloch) (1/1)	<i>Sterrhurus magnacetalulum</i> Guiart, 1938	26°37.95'N; 127°52.00'E	1/1/10	79974
<b>Mullidae</b>				
<i>Parupeneus spilurus</i> (Bleeker) (3/1)	<i>Lecithophyllum hawaiiense</i> Yamaguti, 1970	26°37.95'N; 127°52.00'E	2/1/10	79975
<i>Upeneus tragula</i> Richardson (1/1)	<i>Prosorhynchus</i> sp. <i>Sterrhurus</i> sp.	26°37.95'N; 127°52.00'E	—/—7 1/1/1	79741 79976
<b>Muraenidae</b>				
<i>Echidna delicatula</i> (Kaup) (1/1)	<i>Helicometrina quadrorchis</i> Manter and Pritchard, 1960	26°39.71' to 39.96'N; 127°52.09' to 52.50'E	1/1/5	79882
<i>Gymnothorax flavimarginatus</i> (Ruppell) (1/1)	<i>Aponurus acropomatus</i> Yamaguti, 1938	26°39.71' to 39.96'N; 127°52.09' to 52.50'E	1/1/1	79977
<b>Pomacanthidae</b>				
<i>Centropyge ferrugatus</i> Randall and Burgess (1/1)	<i>Flagellatrema convolutum</i> Ozaki, 1936	26°38.03'N; 127°51.71'E	1/1/7	79978
<i>Centropyge heraldi</i> Woods and Schultz (3/1)	<i>Paragyliauchen arusettae</i> Machida, 1984	26°39.71' to 39.96'N; 127°52.09' to 52.50'E	1/1/5	79979
<b>Pomacentridae</b>				
<i>Amblyglyphidodon cauracae</i> (Bloch) (1/1)	<i>Prosorhynchus</i> sp.	26°37.95'N; 127°52.00'E	1/1/1	79740
<i>Amphiprion clarkii</i> (Bennett) (2/1)	<i>Bucephalopsis ozakii</i> Nagaty, 1937	26°37.95'N; 127°52.00'E	2/1/4	79732
<i>Chrysiptera cyanea</i> (Quoy and Gaimard) (1/1)	<i>Aponurus rhinoplagusiae</i> Yamaguti, 1934	26°37.95'N; 127°52.00'E	1/1/7	79980
<i>Dascyllus aruanus</i> (Linnaeus) (2/1)	<i>Aponurus rhinoplagusiae</i> Yamaguti, 1934	26°37.95'N; 127°52.00'E	2/1/2	79981
<i>Pomacentrus philippinus</i> Evermann and Seale (1/1)	<i>Opcoecius sphaericus</i> Ozaki, 1925 <i>Aponurus rhinoplagusiae</i> Yamaguti, 1934	26°37.95'N; 127°52.00'E	—/—1 1/1/3	79885 79982

TABLE I. Continued.

Hosts (no. examined/no. infected)	Parasite	Locality	No. hosts examined/no. infected/avg. no. worms per infected host	USNM Helm. Coll. No.
Pomadasyidae <i>Plectorhynchus diagrammus</i> (Linnaeus) (1/1)	<i>Prosorhynchus</i> sp.	26°38.03'N; 127°51.71'E	1/1/1	79739
Scorpaenidae <i>Scorpaenopsis diabolus</i> (Cuvier) (3/1)	<i>Bucephalus sphyaenae</i> Yamaguti, 1952	26°39.71' to 39.96'N; 127°59.09' to 52.50'E	1/1/1	79731
Serranidae <i>Epinephelus fasciatus</i> (Forsskal) (3/2)	<i>Brachyphallus parvus</i> (Manter, 1947) <i>Tubulovesicula magnacetabulum</i> Yamaguti, 1939 <i>Opecoelus sphaericus</i> Ozaki, 1925 <i>Sterrhurus</i> sp.	26°39.71' to 39.96'N; 127°59.09' to 52.50'E 26°39.71' to 39.96'N; 127°59.09' to 52.50'E 26°37.95'N; 127°52.00'E 26°37.37'N; 127°51.77'E	1/1/1 2/1/3 3/3/2 2/1/2	79984 * 79983 79884 79987
<i>Epinephelus merra</i> (Bloch) (7/3) <i>Variola albigarinata</i> Baissac (4/2)	<i>Prosorhynchus (Prosorhynchus) platycephali</i> (Yamaguti, 1934) <i>Prosorhynchus (Prosorhynchus) platycephali</i> (Yamaguti, 1934)	26°37.37'N; 127°51.77'E 26°39.71' to 39.96'N; 127°52.09' to 52.50'E	2/1/37 1/1/20	79737 79736
Sphyraenidae <i>Sphyraena barracuda</i> (Walbaum) (1/1)	<i>Sterrhurus</i> sp.	26°37.95'N; 127°52.00'E	1/1/1	79985
Synodontidae <i>Synodus variegatus</i> (Lacepède) (1/1)	<i>Sterrhurus</i> sp.	26°37.95'N; 127°52.00'E	1/1/1	79986



are herein reported from Okinawa for the first time. The flukes include: *Paronatrema mantae* Manter, 1940; *Monolecithotrema kala* Yamaguti, 1970; *Bucephalopsis ozakii* Nagaty, 1937; *Manteriella crassa* (Manter, 1947); *Lecithophyllum hawaiiense* Yamaguti, 1970; *Helicometrina quadorchis* Manter and Pritchard, 1960; and *Brachyphallus parvus* (Manter, 1947).

To our knowledge, the following are new host records: *Preptetos xesuri* (Yamaguti, 1940), in *Acanthurus bleekeri* (Günther); *Lecithophyllum fuscum* Yamaguti, 1938, in *Acanthurus lineatus* (Linnaeus); *Opegaster ditrematis* Yamaguti, 1942, in *Apogon aroubiensis* (Hombron and Jacquinot); *Plagioporus* (*Plagioporus*) *apogonichthydis* Yamaguti, 1938, in *Apogon doederleini* Jordan and Snyder; *Helicometra* (*Helicometra*) *hypodytis* Yamaguti, 1934, in *Cheilodipterus quinquelineatus* Cuvier and *Lethrinus harak* (Forsskål); *Lepocreadium clavatum* in *Rhinecanthus aculeatus* (Linnaeus); *Proisorhynchus* (*Skrjabinella*) *uniporus* Ozaki, 1924, in *Tylosurus crocodilus crocodilus* (LeSuer); *Paragyliuchen chaetodontis* Yamaguti, 1935, in *Plagiotremus tapeinosoma* (Bleeker); *Plagioporus* (*Plagioporus*) *apogonichthydis* in *Cirrhitichthys falco* Randall; *Paradiscogaster chaetodontis okinawensis* Yamaguti, 1971, in *Platax pinnatus* (Linnaeus); *Hamacreadium lethrini* Yamaguti, 1934, in *Lethrinus harak* and *Lutjanus fulviflamma* (Forsskål); *Sterrhurus magnacetabulum* Guiart, 1938, in *Parapercis cylindrica* (Bloch); *Aponurus acropomatis* Yamaguti, 1938, in *Gymnothorax flavimarginatus* (Ruppell); *Flagellotrema convolutum* Ozaki, 1936, in *Centropyge ferrugatus* Randall and Burgess; *Aponurus rhinoplagusiae* Yamaguti, 1934, in *Chrysiptera cyanea* (Quoy and Gaimard); *Dascyllus aruanus* (Linnaeus) and *Pomacentrus philippinus* Evermann and Seale; *Opecoelus sphaericus* Ozaki, 1925, in *Dascyllus aruanus* and *Epinephelus merra* (Bloch); *Bucephalus sphyraenae* Yamaguti, 1952, in *Scorpaenopsis diabolus* (Cuvier); *Tubulovesicula magnacetabulum* Yamaguti, 1939, in *Epinephelus fasciatus* (Forsskål); and *Proisorhynchus* (*Proisorhynchus*) *platycephali* (Yamaguti, 1934) in *Variola albimarginata* Baissac and *V. louti* (Forsskål).

Several specimens of *Bivesicula*, probably *B. epinepheli* Yamaguti, 1938, were found in a single *Epinephelus fasciatus* (Forsskål). Unfortunately, these were inadvertently discarded but not before they were examined.

## DISCUSSION

Yamaguti (1942) reported 51 species of digeneans mainly from marine fishes of Naha, Okinawa. Because of differences in the various groups of fishes examined in his study and the present report and because the prevalence and intensity for each species of digenetic fluke could not be ascertained from the data presented by him, comparison could not be undertaken. Yamaguti described 29 new and reported 21 previously known species, whereas the present study reports only 34 species, none of which is new. In addition to differences above, some of the variance might be attributed to differences in specific localities, as it is well known that parasites abundant in one area may be absent from the same host species only a short distance away (Van Cleave and Mueller, 1934).

In both Yamaguti's study and ours, the majority of infections encountered constituted single infections. The number of hosts with mixed infections decreased as the variety of parasites increased.

Whereas differences exist for the various numerical categories of hosts, both studies reveal that digenetic flukes have a high degree of host specificity in marine fishes. These results substantiate the findings of Manter (1957) who summarized the extent to which digenetic trematodes have been reported from one or more species of marine fishes in Japan, Tortugas, the Mediterranean, and the British Isles, as well as information given by Nahhas and Cable (1964) on host specificity of the digenetic flukes of marine fishes of Curaçao and Jamaica. These results also substantiate the findings of Siddiqi and Cable (1960) and Dyer et al. (1985) for digenetic flukes of marine fishes of Puerto Rico.

The present study presents new geographic localities for 7 digenetic flukes. The phenomenon of geographic distribution of digenetic flukes of marine fishes from widely separated geographic localities has been summarized by Manter (1947, 1955).

An examination of Table I reveals a striking degree of fidelity between the digenetic trematodes and their definitive hosts in Okinawa. Only 3 of 34 species of digenetic trematodes identified in this study have been found to transgress family bounds in their definitive hosts. These are *Plagioporus* (*Plagioporus*) *apogonichthydis* Yamaguti, 1938, *Opecoelus sphaericus* Ozaki, 1925,

and *Hamacreadium lethrini* Yamaguti, 1934. Manter and Van Cleave (1951) likewise reported that only 3 of 17 species of digenetic flukes from marine fishes of La Jolla, California, occurred in 2 or more families of definitive hosts.

More extensive sampling of marine fishes of Okinawa is needed before a digenetic list approaching completeness can be presented.

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#### APPENDIX I

Species of fish negative for digeneans listed alphabetically by family with the number of individuals examined in parentheses

##### Chondrichthyes

Dasyatididae: *Taeniura melanospila* (Bleeker) (1).

##### Osteichthyes

Acanthuridae: *Acanthurus nigrofusus* (Forsskal) (1), *A. olivaceus* Schneider (1); Apogonidae: *Apogon cyanosoma* Bleeker (7), *Cheilodipterus macrodon* (Lacepède) (2), *Rhabdamia gracilis* (Bleeker) (1), *Siphamia versicolor* (Smith and Radcliffe) (4); Atherinidae: *Atherion elymus* Jordan and Starks (2); Aulostomidae: *Aulostomus chinensis* (Linnaeus) (1); Balistidae: *Balistoides conspicillum* (Schneider) (1); Blenniidae: *Ecsenius lineatus* Klauswitz (3), *E. yaeyamaensis* (Aoyagi) (2),

*crolabrichthys pascalus* (Jordan and Tanaka) (2), *Plectropomus leopardus* (Lacepède) (1); Siganidae: *Siganus argenteus* (Quoy and Gaimard) (1), *S. spinus* (Linnaeus) (1); Syngnathidae: *Corythoichthys haematopterus* (Bleeker) (2), *C. schultzi* Herald (1), *Dunckerocampus dactyliophorus* (Bleeker) (1); Synodontidae:

*Saurida gracilis* (Quoy and Gaimard) (1); Tetraodontidae: *Arothron meleagris* (Schneider) (1), *Canthigaster valentini* (Bleeker) (1); Tripterygiidae: *Enneapterygius etheostomus* (Jordan and Seale) (1), *Helicogramma* sp. (5); Zaclidae: *Zanclus cornutus* (Linnaeus) (1).



## EVALUATION OF IMMUNODIAGNOSTIC ANTIGENS IN THE EXCRETORY–SECRETORY PRODUCTS OF *FASCIOLA HEPATICA*

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**ABSTRACT:** The metabolic antigens of *F. hepatica* have been shown to be a source of potential immunodiagnostic antigens. We have fractionated *F. hepatica* excretory–secretory (ES) antigens by conventional gel filtration and HPLC, analyzed these fractions in PAGE, and evaluated their immunogenicity by ELISA with sera from experimentally infected rabbits to identify potential serodiagnostic antigens for fascioliasis. A fraction enriched in high molecular weight components of ca. 150–160 kDa was found to be very reactive with sera from early fascioliasis. This fraction was successfully adapted to the DOT-ELISA, where titers up to 1:16,000 still appeared visually as positive. Both acute and chronic fascioliasis sera also recognized, in the enzyme-linked immunoelectrotransfer blot technique (EITB), prominent 25–30-kDa polypeptides that have previously been shown to be recognized by infected rabbits, cows, and sheep. We have therefore employed conventional gel filtration and HPLC gel exclusion chromatography as a 1-step procedure to obtain fractions enriched in antigens recognized in early fascioliasis. In addition, these antigens have been successfully applied to a sensitive, visual immunodiagnostic technique that can be easily employed in field studies.

Numerous studies have evaluated different antigen preparations and diverse serologic tests for the immunodiagnosis of *F. hepatica* infections. Persistent problems of nonspecificity have encouraged efforts directed toward the isolation of purified and species-specific antigens. *Fasciola hepatica* excretory–secretory products (ES) have been recognized as playing an important role in inducing antibody formation in fascioliasis (Lang and Hall, 1977; Howell and Sandeman, 1979; Rajasekariah et al., 1979; Sandeman and Howell, 1981). The metabolic antigens of *F. hepatica* have also been shown to be a source of potential immunodiagnostic antigens. Various studies have been done with crude or partially purified ES antigens for the serodiagnosis of fascioliasis in both experimental models (i.e., rabbits) and natural hosts (i.e., sheep, cattle). Cuperlovic and Lalic (1972) demonstrated, by complement fixation, that antibodies to metabolic antigens of *F. hepatica* appeared in the circulation of infected animals before clinical and coprological findings were observed. Lehner and Sewell (1980) used metabolic antigens of adult *F. hepatica* in immunodiffusion and ELISA with the sera from sheep, rabbits, and rats and could detect a rise in antibody levels by 2 wk postinfection.

In recent analogous experiments, Pfister et al.

(1984) evaluated 3 somatic and 3 ES antigen preparations using sera from experimentally infected rabbits and naturally infected bovines. Antibodies to ES antigen fractions Fhm 1 and 2 were detected earlier in infection than those to somatic antigens. In recent studies, Santiago et al. (1986) reported that crude *F. hepatica* ES products, when tested by ELISA, had a high reactivity with the sera from rabbits with acute fascioliasis. They used the enzyme-linked immunoelectrotransfer blot to identify various ES polypeptides recognized during an experimental 1-yr *F. hepatica* infection in rabbits.

These studies have encouraged us to continue characterizing these antigens and evaluating their serodiagnostic potential. In the present study, *F. hepatica* ES antigens were fractionated by gel filtration and HPLC and the resulting fractions analyzed by SDS-PAGE and ELISA in order to evaluate the antigenic components in each one and identify potential serodiagnostic antigens for fascioliasis.

### MATERIALS AND METHODS

#### Preparation of *F. hepatica* excretory–secretory products (FhESP)

Live *F. hepatica* adult worms were obtained from infected bovine livers and washed in 0.01 M PBS, pH 7.2, at room temperature. The worms were then incubated (1 worm/5 ml) in PBS containing 0.8 phenylmethylsulfonylfluoride (PMSF) for 3 hr at 37 C. After incubation, the worms were removed and the suspension containing the FhESP centrifuged at 20,000 rpm for 1 hr at 4 C. Protein concentration in the supernatant was measured by BioRad (Bradford, 1976), and the preparation was used fresh. The average protein concentration of the supernatant was 15 µg/ml.

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## Preparation of sera

**Rabbit sera:** *Fasciola hepatica*-infected rabbit sera were obtained from 3 NZW rabbits infected orally with 25 metacercariae of *F. hepatica* and bled repeatedly through 30 wk of infection. Sera were stored in aliquots at  $-20^{\circ}\text{C}$  until used.

Anti-FhESP was prepared by immunizing a rabbit 5 times with 1 ml of FhESP/NRS (1:1 in FIA). FhESP/NRS was prepared by incubating live adult *F. hepatica* worms (3 hr,  $37^{\circ}\text{C}$ ) in normal rabbit serum (1:3 in normal saline) obtained from the same rabbit prior to the inoculation. Normal rabbit serum was added to stabilize the FhESP components from enzyme degradation.

**Mouse sera:** *Schistosoma mansoni*-infected mouse sera were obtained from 40 Swiss Webster female mice infected by subcutaneous injection with 75 cercariae of *S. mansoni*. Mice were bled by the retroorbital vein plexus before infection to obtain normal serum and serially through 13 wk of infection.

## Gel filtration chromatography

*Fasciola hepatica* excretory-secretory products were fractionated using a  $1 \times 120\text{-cm}$  glass column (BioRad, Richmond, California) containing 90 ml of sephadex G-75 (Pharmacia, Piscataway, New Jersey) equilibrated in 0.01 M PBS, pH 7.0, and previously calibrated with known molecular weight markers (Sigma, St. Louis, Missouri). The void volume, determined with blue dextran 2,000 (Sigma), was 20.5 ml. FhESP (6 mg in 10 ml) was applied to the column followed with 3.0 ml of 3% sucrose in PBS/0.02% sodium azide. Fractionation was done by an Isco Wizard peristaltic pump with degassed PBS/0.8 mM PMSF. The elution profile was monitored by absorbance at 280 nm using an Isco UA-5 monitor and recorder. Fractions of 4.2 ml were collected, the protein content was measured (Bradford, 1976), and the fractions were stored at  $-20^{\circ}\text{C}$  until use.

## Fractionation of FhESP by HPLC

FhESP was fractionated in a size exclusion HPLC column (spherogel TSK—4,000 PW,  $7.5\text{ mm} \times 30\text{ cm}$ , Altex-Beckman, San Ramón, California). The proteins were eluted from the column isocratically at  $21^{\circ}\text{C}$ , at a flow rate of 0.5 ml/min. The column was calibrated with a known mixture of molecular weight standards (Pharmacia, Piscataway, New Jersey). All HPLC purification steps were performed on a Waters HPLC system. In a typical experiment, FhESP was run at a concentration of 200  $\mu\text{g}$  protein in 200  $\mu\text{l}$  buffer, and the fractions were collected at 90-sec intervals. The antigenic activity of each fraction was monitored by ELISA using sera from *F. hepatica*-infected rabbits.

## SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out on 5–20% or 10–20% gradient slab gels exactly as described by Tsang et al. (1983). All SDS-PAGE chemicals, including molecular weight markers, were obtained from BioRad (Richmond, California). Gels were cast and run in a  $140 \times 320 \times 0.75\text{-mm}$  vertical slab system from BioRad. All samples were treated with a 10% SDS, 9 M urea, 0.1 M Tris-HCl, pH 8.0, solution to a final concentration

of 2.5% SDS and 1  $\mu\text{g}/\mu\text{l}$  of protein in the final treated sample. Samples were heated at  $65^{\circ}\text{C}$  for 15 min in a water bath and 10–20  $\mu\text{g}$  of protein were added per slot. Molecular weight markers were applied at a concentration of 1  $\mu\text{g}/\mu\text{l}$ , 10  $\mu\text{l}/\text{slot}$ . Gels were stained with Coomassie brilliant blue RF 250 (BioRad).

## Enzyme-linked immunotransfer blot (EITB)

Transfer of proteins from SDS-PAGE to nitrocellulose sheets was performed according to Tsang et al. (1983). Transfer was done at  $4^{\circ}\text{C}$  at constant voltage (60 V) for 3 hr. The nitrocellulose sheet was then removed and washed 3 times with 0.3% Tween 20, 0.1 M PBS, cut into 5-mm strips, and incubated overnight at  $4^{\circ}\text{C}$  in serum diluted 1:200 in PBS/Tween 20. After 3 washes (10 min each) in PBS/Tween 20, the nitrocellulose strips were then incubated in a horseradish peroxidase-conjugated anti-IgG (KPL) 1:1,000 for 2 hr. The strips were then washed as described before and exposed to the substrate solution (3,3'-diaminobenzidine) for 10 min or until the dark brown bands appeared. The strips were then rinsed in water and photographed using an 80 A blue filter.

## Enzyme-linked immunosorbent assay (ELISA)

The different fractions obtained after fractionation by sephadex G-75 were incubated in Falcon polyvinyl EIA plates at a concentration of 3  $\mu\text{g}/\text{ml}$  in 0.6 M carbonate buffer, pH 9.6 (100  $\mu\text{l}/\text{well}$ ) and incubated at  $4^{\circ}\text{C}$  for 24 hr. After incubation, unadsorbed antigen was removed by inverting the plates and washing 3 times for 3 min each with 0.05% Tween 20, 0.1 M PBS. All sera were diluted (1:128) in PBS/Tween 20, added to the wells (100  $\mu\text{l}/\text{well}$ ), and incubated for 1 hr at  $37^{\circ}\text{C}$ . The sera were then removed and the plates washed as described above. Horseradish peroxidase-labeled anti-IgG antibody (100  $\mu\text{l}$  at a 1:1,000 dilution in PBS/Tween 20) was added to each well and incubated for 1 hr at  $37^{\circ}\text{C}$ . The conjugate was removed and the plates washed again as described above. The substrate (orthophenylenediamine-OPD) was then added to each well (100  $\mu\text{l}$ ), and the plates were incubated in the dark for 30 min; the reaction was terminated with 25  $\mu\text{l}$  8 N  $\text{H}_2\text{SO}_4$  and the plates read at 490 nm in a Dynatech Minireader II. Runs were done in triplicate and basic statistical analysis was applied using the titration and absorbance method summarized by Savigny and Voller (1980).

## DOT-ELISA

All reagents, materials, and equipment were obtained from BioRad (Richmond, California). Nitrocellulose sheets were soaked in TBS buffer (20 mM Tris, 500 mM NaCl, pH 7.5) for 10 min and mounted in the Bio-Dot microfiltration apparatus. TBS (100  $\mu\text{l}$ ) was vacuum-filtered through the nitrocellulose membrane. Antigen fractions were then added (1  $\mu\text{g}$  in 100  $\mu\text{l}$  TBS) and allowed to filter through the membrane by gravity. After filtration, wells were vacuum-dried and washed 3 times with 100  $\mu\text{l}$  of washing solution (TBS, 0.25% Tween 20, pH 7.5). The sera were diluted serially (starting at 1:256) in TBS, applied to the plates (100  $\mu\text{l}/\text{well}$ ), and allowed to filter through the membrane by gravity. Wells were then dried and washed as above. The nitrocellulose sheets were removed from



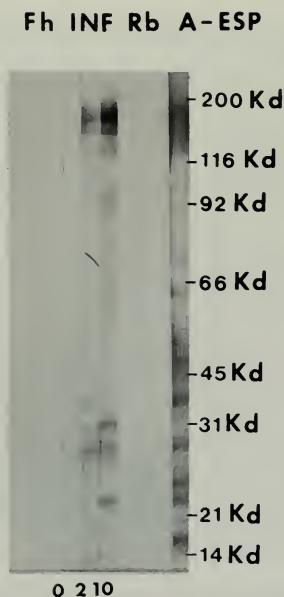


FIGURE 1. EITB of FhESP reacted with normal rabbit serum (0 wk) and the serum from a rabbit infected with *F. hepatica* and bled at 2 and 10 wk. The serum from a rabbit immunized with the FhESP antigens (A-ESP) was also tested.

the Bio-Dot apparatus, washed for 15 min in PBS, and exposed to the substrate solution (50 mg of 3,3'-diaminobenzidine, 10  $\mu$ l 30%  $H_2O_2$  in 100 ml of PBS, pH 7.2) for 10 min.

## RESULTS

### EITB analysis of crude FhESP antigens

When FhESP are collected in the presence of PMSF and analyzed in the EITB with early fascioliasis serum, 2 main clusters of polypeptides are detected: one very prominent cluster of 25–30 molecular weight polypeptides and an additional

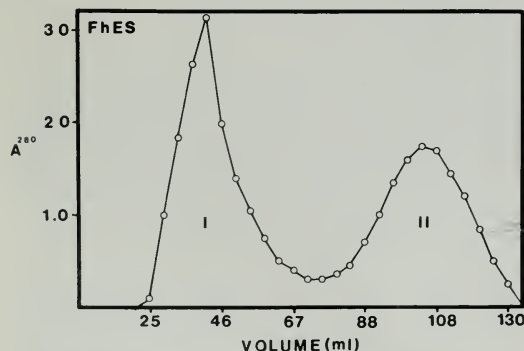


FIGURE 2. Elution profile of sephadex G-75 fractionation of FhESP.

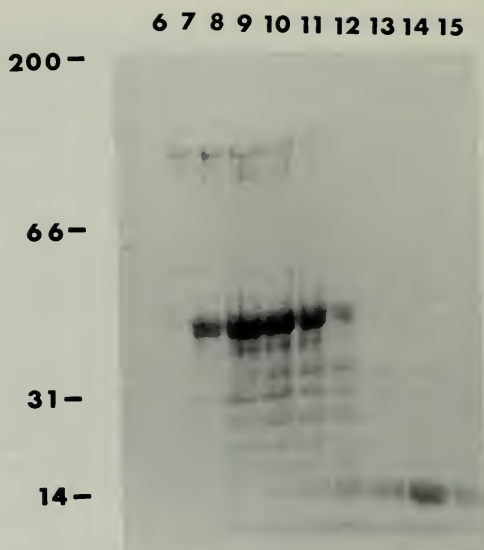


FIGURE 3. SDS-PAGE of FhESP fractions obtained by gel filtration chromatography. Numbers at the top denote fraction number. Each well contained 50  $\mu$ g total protein; the gel was stained with Coomassie blue.

group of 150–160 kDa. By 10 wk of infection, however, additional antigenic polypeptides of 95, 66, and 23 kDa are clearly observed. The serum from the rabbit immunized with the FhESP recognizes at least 8–10 additional polypeptides, from 14 to 66 kDa, which are not seen by the infected animals (Fig. 1).

### Fractionation of FhESP antigens by gel filtration

When crude FhESP was fractionated on a sephadex G-75, 2 main peaks of material eluted from the column (Fig. 2). The first peak (fractions 6–12) eluted at the void volume and by SDS-PAGE was shown to have fractions enriched in high molecular weight components of 150–160 kDa, fractions enriched in medium size polypeptides of 25–48 kDa, and fractions in the descending portions, enriched in 12–14-kDa molecules (see Fig. 3). Peak II (fractions 13–15) contained low molecular weight peptides (12 kDa).

### Serologic evaluation of FhESP by ELISA and DOT-ELISA

The fractions described above were tested in ELISA with sera from a rabbit with early fascioliasis (Fig. 4). Although most fractions of peak I were clearly reactive in ELISA with the serum from 5 wk postinfection, serum from 3 wk only showed strong reactivity with the fractions en-



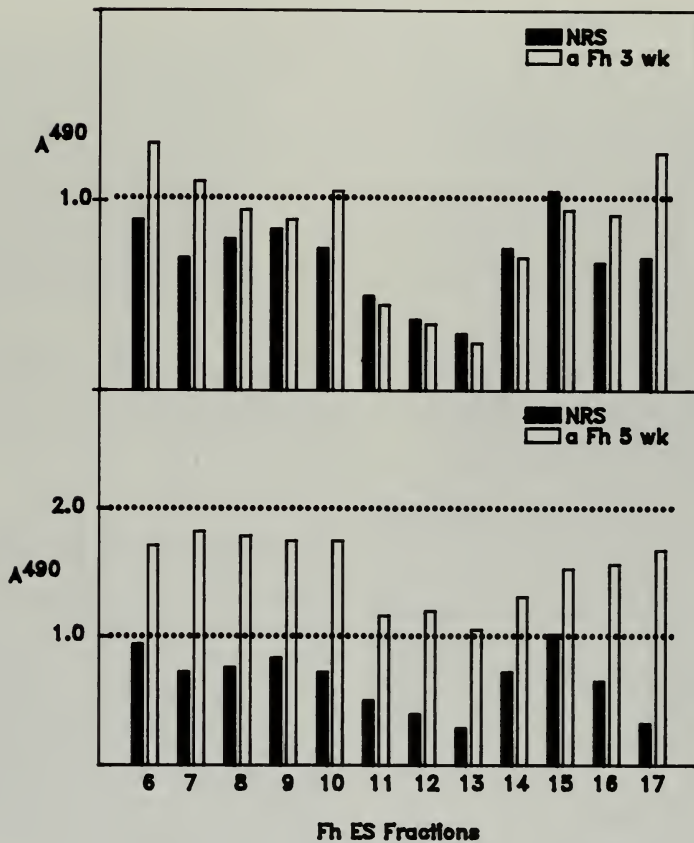


FIGURE 4. ELISA absorbance profile comparing the reactivity of the FhESP fractions (6–17) reacted with normal rabbit serum (NRS), serum from a rabbit infected with *F. hepatica* for 3 wk (a-Fh 3 wk; top panel), and for 5 wk (a-Fh 5 wk; bottom panel). All sera were tested at a 1:128 dilution.

riched in the higher molecular weight components (150–160 kDa) and the low molecular weight peptides of 12–14 kDa. The components of the second peak (12 kDa) were found to be nonreactive with the sera from rabbits with fascioliasis.

We also evaluated these fractions in the DOT-ELISA, testing all the fractions in peak I with acute and a 5-wk chronic fascioliasis sera. Although the serum from chronic infection (Fig. 5, lower panel) reacted strongly with fractions enriched in the 25–30-kDa antigens (fractions 9 and 10) as well as fractions enriched in the 150–160-kDa molecules (fractions 6–8), and those containing the 12–14-kDa molecular weight polypeptides (fractions 14, 15); the serum from early fascioliasis has a strong reactivity with the fractions enriched in the 150–160-kDa polypeptides (Fig. 5, top panel, fraction 7). When this fraction enriched in the 150–160-kDa antigens was re-

acted in the DOT-ELISA with the titrated serum from a rabbit bled through 21 wk, not only is reactivity detected by 3 wk, but later in infection titers of up to 1:16,000 still appear visually as positive (Fig. 6).

#### Fractionation of FhESP antigens by size exclusion HPLC

Size exclusion HPLC was used as a first step in the purification of target antigens. A typical elution profile is seen in Figure 7 along with the corresponding ELISA activity associated with each peak. Approximately 10 peaks were detected, all of which reacted with the sera from infected rabbits. Peaks with retention times of 20 and 28 min were shown to be enriched, respectively, in 150–160- and 25–30-kDa polypeptides, both of which were recognized by an early *F. hepatica* infection serum (Fig. 8).

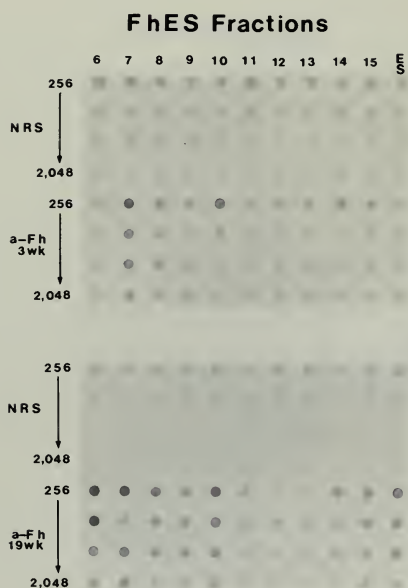


FIGURE 5. DOT-ELISA comparing the reactivity of crude FhESP and of the different FhESP fractions (1  $\mu$ g/ml) obtained by gel filtration chromatography reacted with normal rabbit serum (NRS) and 3-wk *F. hepatica*-infected rabbit serum (a-Fh 3 wk). All sera were diluted serially starting at 1:256 to 1:2,048.

## DISCUSSION

The identification and isolation of antigens is of fundamental importance not only to obtain a better understanding of mechanisms of immunity, but to facilitate the preparation of purified, specific antigens suitable for immunodiagnosis. The present study aimed to identify and obtain in reasonable quantity antigens that could offer improved sensitivity and specificity in the serodiagnosis of fascioliasis.

Most of the studies done on the application of ES antigens for serodiagnosis have employed crude ES antigen preparations. Various groups have, however, evaluated partially purified preparations.

Lehner and Sewell (1980) fractionated ES antigens by sephadex G-200 and obtained an elution profile of 2 peaks, the second of which was very disperse. When the individual fractions were tested in ELISA with sera from infected sheep, rats, and rabbits, they found antigenic activity mainly in the higher molecular weight fractions for all 3 species. Whereas rabbits and sheep also showed reactivity with other lower molecular weight components, infected rat serum reacted only with these high molecular weight fractions.

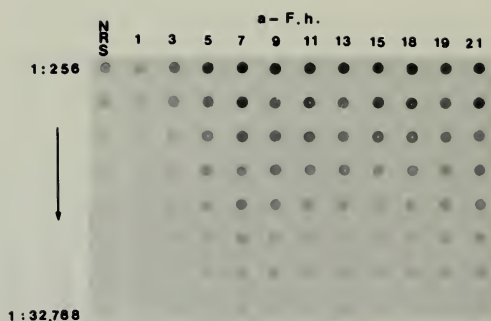


FIGURE 6. DOT-ELISA of FhESP fraction 7 (1  $\mu$ g/ml) containing the 5 polypeptides of 150–160 kDa tested with NRS and sera from the rabbits infected through 21 wk of infection. All sera were diluted from 1:256 up to 1:32,768.

Our results, using sera from experimentally infected rabbits, confirm this observation. Similar studies were done by Pfister et al. (1984), who evaluated 2 ES antigen preparations, those >10 kDa and those from 2 to 10 kDa. Antigenic activity was found in both antigen preparations; however, improved specificity was observed in the fraction with components >10 kDa. In our study, antigenic components were identified in all the fractions tested, but early fascioliasis was detected by the higher molecular weight fractions (150–160 kDa).

In recent studies (Santiago et al., 1986) 25–30-kDa polypeptides were the most prominently recognized in EITB with the sera of rabbits with acute fascioliasis. These antigens are also clearly detected in our EITB's with rabbit sera. Studies with the serum from *F. hepatica*-infected sheep agree with our results. Thus, Irving and Howell (1982) found polypeptides of 23, 24, and 26 kDa as the 3 major labeled components detected in the culture media of juvenile *F. hepatica* incubated in [ $^{14}$ C] leucine. These 3, plus a 27-kDa polypeptide, were precipitated with sera from *F. hepatica*-infected sheep. Zimmerman and Clark (1986) employed both conventional molecular exclusion chromatography and FPLC protein fractionation systems to analyze ES products from adult *F. hepatica*. They monitored the antigenic activity of fractions obtained by superose-6 FPLC fractionation of *F. hepatica* ESP with sera from normal and infected sheep in the DOT-ELISA. As in the present study, not only did they detect antigens reactive with infected sheep sera with molecular weights of  $\sim$ 24 kDa, but they demonstrated other sensitive immunoreactive com-

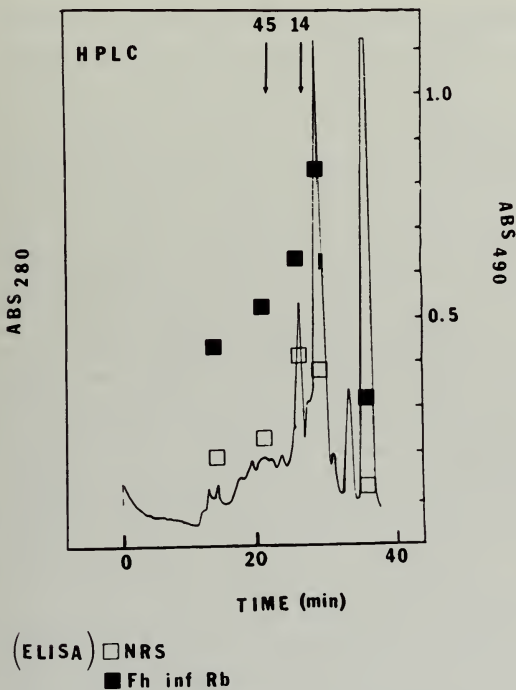


FIGURE 7. Elution profile of the fractionation of FhESP in a spherogel TSK-4,000 PW column in HPLC. Open squares represent ELISA absorbance values of normal rabbit serum and closed squares the values of serum from a rabbit infected with *F. hepatica* for 8 wk. Numbers at the top are 2 molecular weight standards.

ponents in the higher molecular weight ranges. They also detected some nonspecific cross-reactivity of some higher molecular weight components within the 81–200-kDa region when reacted with normal sheep serum. The fact that the prominently detected 150–160-kDa polypeptides identified in the present study were not detected in previous studies may be due to the lack of protease inhibitors in these antigen preparations, which may cause the 150–160-kDa antigenic polypeptides to be degraded into lower molecular weight components. The fact that proteolytic activity has been demonstrated in homogenates and ES products of *F. hepatica* further supports this hypothesis (Thorsell and Bjorkman, 1965; Locatelli and Beretta, 1969; Simpkin et al., 1980).

It has been shown, not only in fascioliasis, but in other parasitic infections as well, that there are differences in the antigenic molecules that different experimental hosts will recognize in the same postinfection periods, and that there may also be variations in the antigens recognized by the same host during infection. From this view-

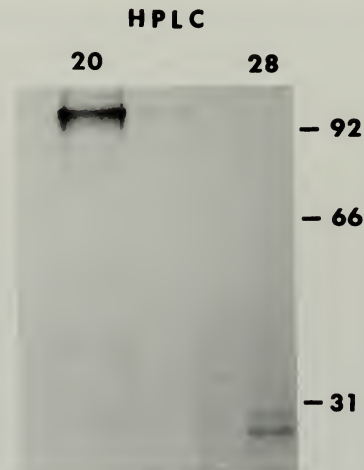


FIGURE 8. EITB of selected fractions obtained by HPLC fractionation and reacted with serum from a rabbit infected with *F. hepatica* for 10 wk. Numbers at the top denote retention times; numbers at the right indicate molecular weight standards.

point the 25–30-kDa polypeptides constitute excellent candidates for acute fascioliasis, because previous work has shown them to be consistently recognized by rabbits, cows, and sheep infected with this trematode. In terms of detecting early fascioliasis, however, the fractions enriched in 150–160-kDa antigens appear to offer the best sensitivity. Because none of the above-described fractions showed reactivity with sera from infection with *Schistosoma mansoni*, a related trematode, improved immunologic specificity can also be expected.

In conclusion, in the present study we have identified ES antigens with approximate molecular weights of 150–160 kDa that are recognized by early fascioliasis serum. Both conventional liquid chromatography using sephadex G-75 and gel exclusion HPLC provide 1-step procedures to obtain fractions enriched in these antigens. Upscaling the isolation procedure to a preparative mode could provide an efficient method of obtaining improved yields of these antigens.

#### ACKNOWLEDGMENTS

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## MONOCLONAL ANTIBODIES REVEAL ANTIGENIC DIFFERENCES IN REFRACTILE BODIES OF AVIAN *EIMERIA* SPOROZOITES

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**ABSTRACT:** Monoclonal antibodies were developed against refractile body antigens of 4 species of avian *Eimeria*, *E. meleagritidis*, *E. adenoides*, *E. acervulina*, and *E. tenella*. Although antibodies from 8 different cell lines were used in this study, all produced similar fluorescent and gold-labeling patterns. By immunofluorescent antibody techniques, 5 of the 8 antibodies cross-reacted with all 4 of the *Eimeria* species that were examined; the other 3 antibodies reacted only with the species against which they were produced or with a limited number of species. In Western blot analyses using SDS-solubilized sporozoites as antigen, 4 of the cross-reactive antibodies recognized multiple bands; the predominant bands had molecular weights of approximately 23, 45, and 90 kilodaltons (kDa). Two of the antibodies with more limited reactivity recognized either a single band at 23 kDa (91C7), or bands at 23 and 45 kDa (4115); another reacted only with several bands >100 kDa (4D10). The molecular weights of the antigens did not decrease markedly after digestion with N-glycanase F, indicating that if the refractile body antigens contained significant amounts of N-linked carbohydrate it was refractory to the enzyme. Collectively, the data indicate that antigens of the sporozoite refractile bodies differ among the *Eimeria* species. Some antigens are conserved, whereas others differ in distribution or frequency among the individual species.

Refractile bodies (RB) are the most conspicuous structures in the sporozoites of most *Eimeria* species, occupying approximately 30–50% of the cytoplasmic area. These structures have been described as clear globules (Hammond, 1973) that are proteinaceous and have no indication of structure (Ryley, 1973). The function of the RB is unknown. In the species of avian *Eimeria* that have been studied, the RB appear to undergo changes in number, shape, and location during the first few days after invading a host cell (Fayer, 1969). Investigators have speculated that the RB, because of the rapid changes after entering a cell, may play a role in the early development of the parasite (Chobotar and Scholtyseck, 1982). A recent study has indicated that refractile body antigens of *E. tenella* must diffuse throughout the first-generation schizont for maturation to occur. Collectively, these studies suggest that inhibition of RB activity might result in decreased intracellular development of the parasite. The RB antigens of sporozoites of the avian *Eimeria* species have not been described. In the study reported here, RB antigens of 4 species of avian *Eimeria*, *E. meleagritidis*, *E. adenoides*, *E. acervulina*, and *E. tenella*, were examined. Monoclonal antibodies generated against the RB were used as probes for differences

in distribution, molecular weight, and in the extent of N-linked glycosylation.

### MATERIALS AND METHODS

#### Sporozoites

Sporozoites of *Eimeria meleagritidis* and *E. adenoides* (species that infect turkeys) and *E. tenella* and *E. acervulina* (species that infect chickens) were excysted, cleaned of debris (Danforth, 1982), and subjected to the following procedures: (1) for indirect immunofluorescent antibody (IFA) tests, sporozoites of each species were air-dried on 12-well teflon-coated slides (Danforth, 1982); (2) for determination of alcohol stability, air-dried sporozoites of *E. tenella* were treated with cold (4°C) 100% ethanol or methanol for 2 min and washed with phosphate-buffered saline, pH 7.4, for 2 min; (3) for gold-labeling experiments, sporozoites of *E. meleagritidis* and *E. tenella* were fixed in 3% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 1 hr at room temperature (RT;  $22 \pm 2^\circ\text{C}$ ) and washed in cacodylate buffer, pH 7.2, for 10 min; and (4) for Western blot analysis with or without N-glycanase F digestion, sporozoites of each species were suspended in 0.5 ml of sample buffer (Laemmli, 1970), disrupted by vortexing with glass beads (100  $\mu\text{m}$  in diameter) for 3 min, and boiled for 3–5 min. The same numbers of sporozoites were similarly homogenized in PBS and the protein levels were measured using the BCA Micro Protein Assay System (Pierce Chemical Company, Rockford, Illinois). Based on this protein assay, the volume of sample buffer was adjusted so that the antigen preparations of the 4 species that were applied to the gels contained approximately the same concentration of protein.

#### Monoclonal antibodies

Monoclonal antibodies (McAb) that react specifically with the RB of sporozoites were produced as pre-

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TABLE I. *Characteristics of monoclonal antibodies (McAb) that react with refractile bodies of sporozoites of avian Eimeria.*

McAb	Eliciting species	Ig isotype	Cross-reactive with*			
			<i>E. meleagridis</i>	<i>E. adenoides</i>	<i>E. acervulina</i>	<i>E. tenella</i>
1209	<i>E. acervulina</i>	G2a	+	+	+	+
53A8	<i>E. meleagridis</i>	G2b	+	+	+	+
1A12	<i>E. acervulina</i>	G2a	+	+	+	+
45C7	<i>E. meleagridis</i>	M	+	+	+	+
B3	<i>E. tenella</i>	G2b	+	+	+	+
4115†	<i>E. maxima</i>	G2b	—	—	—	+
91C7	<i>E. adenoides</i>	G2a	+	+	—	—
4D10	<i>E. adenoides</i>	M	+	+	—	+

\* As determined by indirect immunofluorescent antibody tests on air-dried sporozoites.

† Elicited against *E. maxima* but, of the 4 species used in this study, cross-reacted with only *E. tenella*.

viously described (Danforth, 1982; Danforth and Augustine, 1983). The isotypes of the McAb were determined with a mouse monoclonal subtyping kit (HyClone Laboratories, Logan, Utah). For cross-reactivity and Western blot studies, each culture supernatant was diluted with Dulbecco's minimum essential medium (DMEM) so that a 1:2 dilution yielded a positive IFA reaction on homologous, air-dried sporozoites and a 1:4 dilution produced only very slight fluorescence. For gold labeling, the supernatant fluid was concentrated 5× with Amicon Macrosolute Concentrators (Amicon Corporation, Danvers, Massachusetts).

#### IFA reactivity of McAb

The reactivity of each McAb with RB of the 4 species of *Eimeria* was determined by IFA procedures on air-dried sporozoites (Danforth, 1982). In addition, the reactivity of RB of ethanol- and methanol-treated *E. tenella* sporozoites with McAb 1209, 4D10, and B3 was compared with that of DMEM-treated sporozoites. Slides were examined with a Zeiss Ultraphot microscope equipped for epifluorescence microscopy.

#### Gold labeling

Paraformaldehyde/glutaraldehyde-fixed sporozoites were dehydrated in a graded series of ethanol (70–100%) and embedded in LR White resin (Polysciences, Inc., Warrington, Pennsylvania). After polymerization for 24 hr at 60 C, the specimens were sectioned on a Sorval MT-2 ultramicrotome and mounted on nickel grids. The sections were exposed to McAb and then to goat antimouse immunoglobulins conjugated with 15-nm gold particles (E-Y Labs Inc., San Mateo, California) using the procedure of Timms (1986). The specimens were examined on a Philips 200B electron microscope.

#### N-glycosidase F digestion

Sporozoite homogenate (10 µl) was combined with 10.8 µl of 0.55 M NaPO<sub>3</sub> (pH 8.6), 3 µl 100 nM 1,10 phenanthroline hydrate, 5 µl 7.5% NP40, and 1.2 µl (1.2 units) N-glycanase F (Genzyme, Inc., Boston, Massachusetts). The mixture was digested for 15 hr at 37 C. For nondigested controls, similar amounts of sporozoite protein were incubated as described above

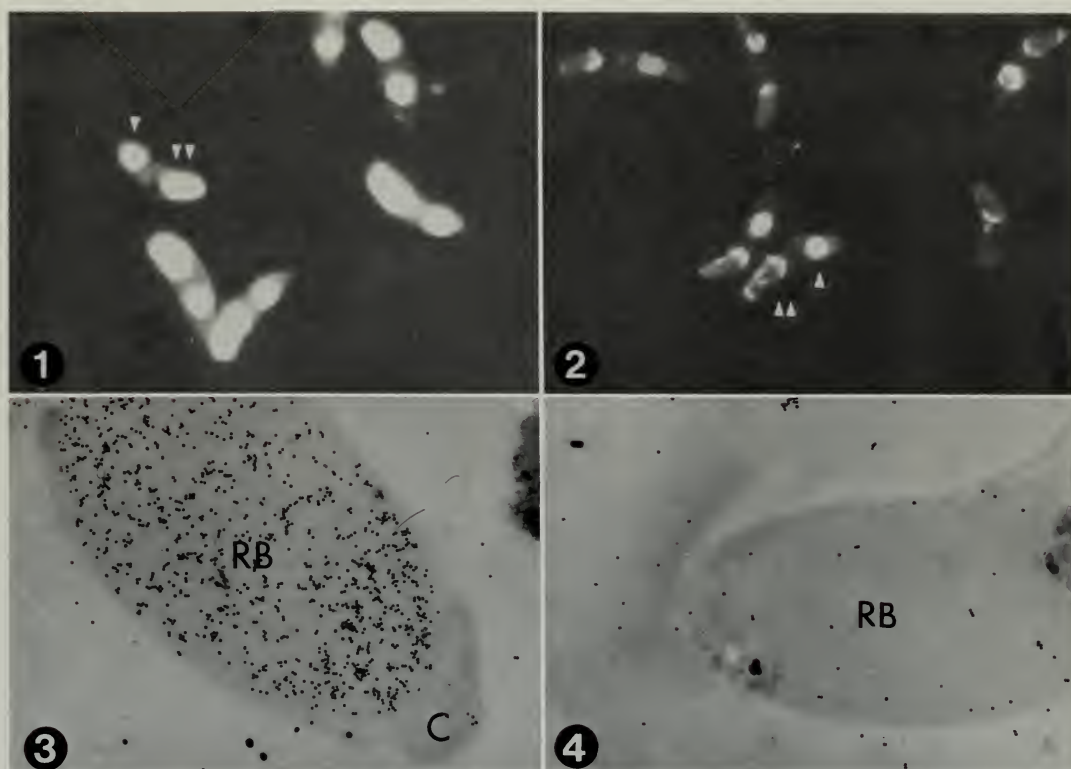
except that N-glycanase F was not added to the reaction mixture.

#### Gel electrophoresis and Western blot analysis

Three experiments were run with 2 or 3 replicates per experiment. In Experiment 1, a single species of *Eimeria* was run on each gel, electrophoretically transferred, and exposed to a battery of 8 McAb. In Experiment 2, all 4 species of *Eimeria* were run on each gel and exposed to a single McAb. In both experiments, 4 µl of sporozoite homogenate, containing from 3.3 to 5.4 µg of protein, was applied to each well and subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) at 100 V/60 mA for 1.5 hr at 4 C, using 11% polyacrylamide (SDS-PA; 33:1 acrylamide:bisacrylamide) gels and 5% stacking gels. The separated proteins were electrophoretically transferred onto nitrocellulose paper at 35–39 V/20 mA for 2 hr at 4 C. In Experiment 3, N-glycanase F-digested or nondigested sporozoite protein was electrophoretically separated on 12.5% SDS-PA gels and transferred onto nitrocellulose paper at 30 V for 15 hr. Prestained protein standards (BRL, Gaithersburg, Maryland), which included myosin—200,000; phosphorylase B—97,400; bovine serum albumin—68,000; ovalbumin—43,000; chymotrypsinogen—25,700; B lactoglobulin—18,400; and lysozyme—14,300, were electrophoresed and transferred along with the sporozoite protein.

The nitrocellulose sheets from the 3 experiments were subjected to Western blot procedures using a modification of the method described by Towbin et al. (1979). Briefly, the nitrocellulose was blocked in gelatin (3% in Tris-buffered saline, pH 7.5) overnight at RT. The paper was rinsed twice (15 min each) with distilled water, cut into strips, and exposed to the McAb or control solutions (culture supernatants without McAb) for 5 hr at RT. The strips were rinsed twice (15 min each) in phosphate-buffered saline containing 0.05% Tween 20 (PBS/T20) and exposed to rabbit antimouse IgG (1:1,000 in antibody buffer; Miles Laboratories, Elkhart, Indiana) for 2 hr at RT. After 2 15-min washes in PBS/T20, the strips were incubated in goat anti-rabbit IgG (1:1,000 in antibody buffer; Miles Laboratories) for 2 hr at RT and then washed again with PBS/T20. The nitrocellulose paper was developed with the Peroxidase Substrate System (4-chloro-1-naphthol;





FIGURES 1–4. Immunofluorescent (IFA) or ultrastructural gold labeling of sporozoites of avian *Eimeria* species after exposure to monoclonal antibodies directed against refractile body antigens. 1. IFA of *E. meleagridis* exposed to McAb 1209. Note uniform fluorescence of anterior (arrowhead) and posterior (double arrowhead) refractile bodies.  $\times 1,062$ . 2. IFA of methanol-treated *E. tenella* exposed to McAb 1209. Note uniform fluorescence of anterior refractile body (arrowhead) and localization of fluorescence of posterior refractile body (double arrowhead).  $\times 1,062$ . 3. Immunogold labeling of *E. meleagridis* exposed to McAb 4D10. Note gold label on refractile body (RB) and absence of label on cytoplasm (C).  $\times 25,000$ . 4. Immunogold labeling of *E. tenella* exposed to McAb B3. Note absence of gold label on refractile body (RB).  $\times 25,000$ .

Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Maryland) for 5–15 min.

## RESULTS

### Monoclonal antibodies

Approximately 150–200 McAb were produced against each *Eimeria* species. When sporozoites of *E. meleagridis*, *E. adenoides*, or *E. tenella* were used as the immunogen, 3–5% of the McAb that were produced reacted specifically with the RB; with *E. acervulina* as the immunogen, about 15% of the McAb was RB-specific. RB McAb reacting with each species were selected for this study. Two each were elicited against *E. meleagridis*, *E. adenoides*, and *E. acervulina*, and 1 against *E. tenella*. The eighth McAb was elicited against *E. maxima*, but, of the species of *Eimeria* used in this study, it reacted only with

*E. tenella*. The isotypes of the RB antibodies included immunoglobulins G and M (Table I).

### IFA and gold labeling

All 8 McAb produced positive IFA reactions with the anterior and posterior RB of air-dried sporozoites (Fig. 1). When sporozoites were treated with 100% ethanol before they were subjected to the IFA procedure, no change in the reactivity of McAb 1209 and 4D10 was observed. However, after sporozoites were treated with 100% methanol, there was a marked reduction in the reactivity of these 2 McAb; the reaction appeared to be localized primarily in the anterior RB and at the anterior end of the posterior RB (Fig. 2). No IFA reaction was observed with McAb B3 after the sporozoites were treated with either ethanol or methanol. Similarly, with gold-labeling procedures, McAb 1209

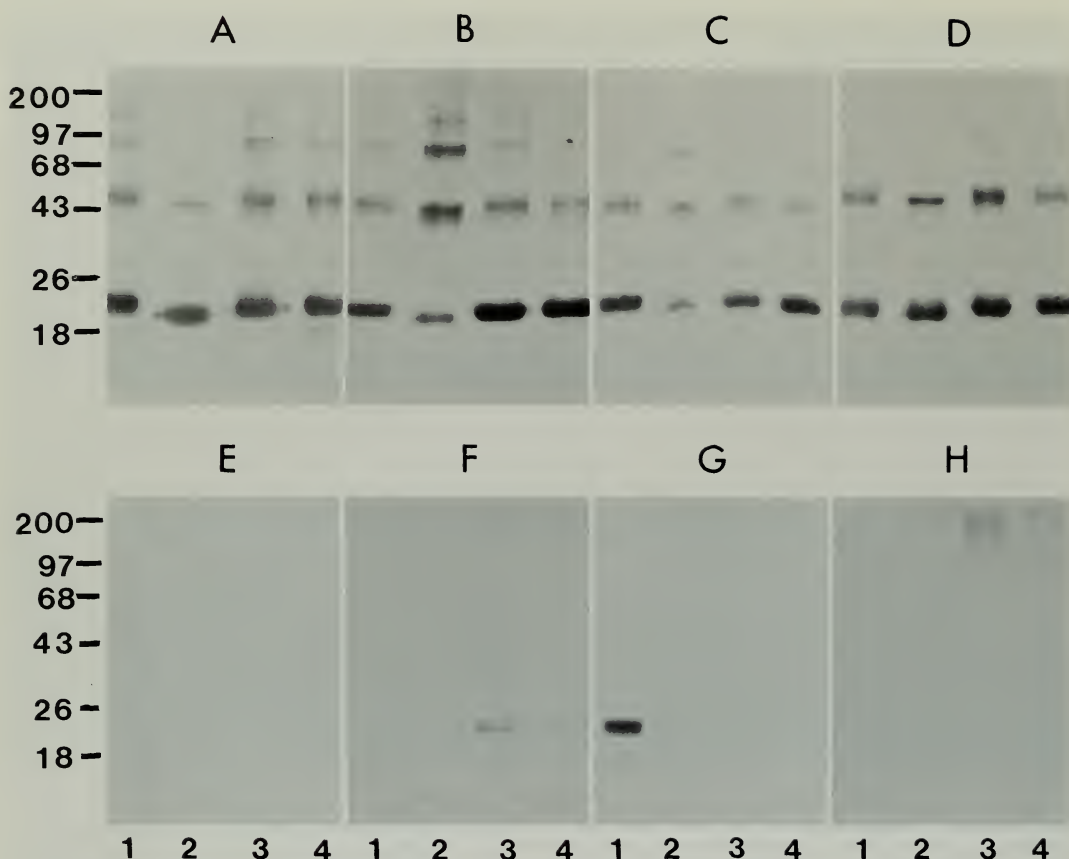


FIGURE 5. Western blot analysis (Experiment 2) of SDS-solubilized sporozoites of *E. tenella* (lane 1), *E. acervulina* (lane 2), *E. adenoeides* (lane 3), and *E. meleagrimitis* (lane 4). Each blot (A-H) contained the 4 *Eimeria* species and was exposed to a different monoclonal antibody: 1209 (A), 45C7 (B), 1A12 (C), 53A8 (D), B3 (E), 91C7 (F), 4115 (G), and 4D10 (H).

and 4D10 specifically labeled the RB (Fig. 3), but McAb B3 did not (Fig. 4).

#### Cross-reactivity

Of the 8 McAb used in this study, 5 (1209, 53A8, 45C7, 1A12, and B3) cross-reacted with all 4 species of *Eimeria* as determined by IFA procedures on air-dried sporozoites (Table I). However, 1 (91C7) reacted with only the 2 species of *Eimeria* that infect the turkey, 1 (4115) reacted only with *E. tenella*, and 1 (4D10) reacted with the 2 species of turkey *Eimeria* and very slightly with *E. tenella* (Table I).

#### Western blot analysis

In Experiments 1 and 2, similar results were obtained; the blots from Experiment 2 are shown in Figure 5. Four of the 5 McAb that cross-reacted in the IFA procedures (1209, 45C7, 1A12,

and 53A8) also cross-reacted in the Western blot analysis (Fig. 5A-D). These 4 McAb reacted with major bands having molecular weights (MW) of approximately 23, 45, and 90 kDa. McAb 1209, 45C7, and 1A12 reacted also with several bands >100 kDa (Fig. 5A-C). The molecular weight (MW) of the smallest major antigen recognized by McAb 1209 appeared to be lower for *E. acervulina* than for the other species (20 kDa vs. 23 kDa; Fig. 5A, lane 2). The fifth McAb that cross-reacted by IFA techniques (B3) failed to react with any of the antigens by Western blot (Fig. 5E).

The 3 McAb that showed limited reactivity in the IFA procedures (91C7, 4115, and 4D10) also demonstrated limited reactivity by Western blot analysis and, in addition, recognized fewer bands. For example, McAb 91C7 reacted with only the 2 turkey species, *E. adenoeides* and *E. mele-*

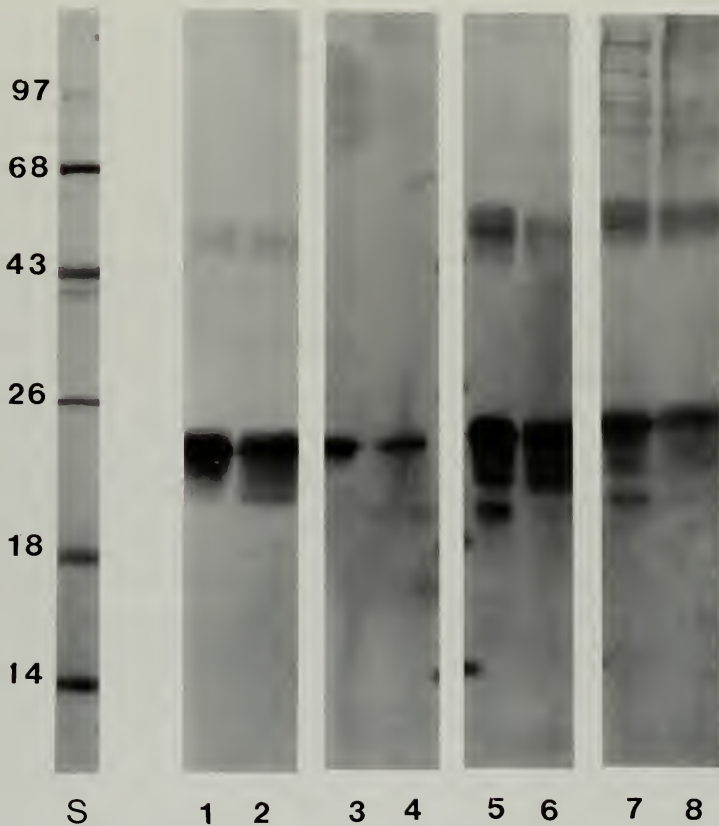


FIGURE 6. Western blot analysis (Experiment 3) of SDS-solubilized sporozoites of *E. tenella*. Lanes 1 and 2 exposed to McAb 1A12; lane 1 nondigested, and lane 2 digested with N-glycanase F. Lanes 3 and 4 exposed to McAb 4115; lane 3 nondigested, and lane 4 digested with N-glycanase F. Lanes 5 and 6 exposed to McAb 53A8; lane 5 nondigested, and lane 6 digested. Lanes 7 and 8 exposed to McAb 45C7; lane 7 nondigested, and lane 8 digested.

*agrimonitis*, by IFA and recognized a single 23-kDa band in each of these species by Western blot analysis (Fig. 5F). Similarly, McAb 4115 reacted with only *E. tenella* by IFA and with *E. tenella* antigens of 23 and 45 kDa (Fig. 5G). In contrast, the antigens recognized by McAb 4D10 differed markedly from those recognized by the other McAb. The prominent antigens, 23, 45, and 90 kDa, were not labeled by McAb 4D10. Instead, only several bands with MW > 100 kDa were observed (Fig. 5H).

In Experiment 3, incubation of sporozoite protein with N-glycanase F did not appear to significantly or consistently reduce the MW of *E. adenoeides* or *E. tenella* RB antigens that were recognized by any of the McAb (Fig. 6). With McAb 1A12 and 4115, new or stronger bands (MW = ~18–20 kDa) were observed in the digested samples (Fig. 6, lanes 2, 4) that were not in the nondigested samples (Fig. 6, lanes 1, 3).

However, when probed with McAb 53A8 and 45C7, bands (MW = ~20 kDa) present in the nondigested samples (Fig. 6, lanes 5, 7) were not seen in the digested ones (Fig. 6, lanes 6, 8).

#### DISCUSSION

Indirect fluorescent antibody, gold-labeling, and Western blot techniques using RB-specific McAb indicated that the RB of sporozoites of the avian *Eimeria* species differ in their antigenic composition. Five of the 8 McAb used in this study reacted with RB antigens that were conserved among the 4 species of *Eimeria*, whereas the other 3 McAb reacted with RB antigens that appeared to be more limited in distribution among the *Eimeria* or in quantity within the individual species. One of the McAb (91C7) reacted only with the 2 species of *Eimeria* that infect the turkey and not with the species that infect chickens.



There was a correlation between the distribution of a specific antigenic determinant among the *Eimeria* species as determined by IFA and the number of polypeptide bands that were detected by Western blot analysis. When subjected to IFA, 5 of the 8 McAb reacted with the RB of air-dried sporozoites of each *Eimeria* species. Western blot analysis showed that 4 of these McAb reacted with 3–6 bands ranging from ~23 kDa to >100 kDa (Fig. 5A–D). The fifth cross-reactive McAb, B3, failed to react by Western blot analysis (Fig. 5E). The prominent bands, 23, 45, and 90 kDa, and perhaps the higher MW bands, may represent multimeric forms derived from a single RB protein.

The McAb that reacted with only 1 or 2 of the *Eimeria* species by IFA recognized fewer bands in the Western blot (Fig. 5F–H) than did the cross-reactive McAb. McAb 91C7 showed a positive IFA reaction only with the 2 turkey species of *Eimeria* and recognized a single 23-kDa band (Fig. 5F); McAb 4115 reacted with only *E. tenella* and recognized bands at 23 and 45 kDa (Fig. 5G). Because the antigenic determinants recognized by these 2 McAb were not detected in all 4 species of *Eimeria*, they probably differ from those recognized by the cross-reactive McAb. The labeling of fewer bands also suggests that the determinants recognized by 91C7 and 4115 may be less abundant than those recognized by the cross-reactive McAb. The sporozoite antigens for Western blots were prepared by the same procedures and were similar in protein concentration; the McAb were diluted to give similar labeling intensities on homologous, air-dried sporozoites. Therefore, the absence of the higher MW bands probably reflects differences in the distribution or frequency of the individual determinants and not the amount of protein applied to the gel or to antibody concentration.

Exposure to alcohols produced changes in the interactions between some of the McAb and their determinants. McAb 1209 and 4D10 continued to react with methanol-treated sporozoites, although the reactivity was decreased from that of untreated sporozoites (Figs. 1, 2), and to label bands in the Western blot analyses (Fig. 5A, H). However, McAb B3, although it intensely labeled air-dried sporozoites, failed to label alcohol-treated ones in either the IFA or gold-labeling procedures (Fig. 4) and failed to react with any electrophoretically transferred bands (Fig. 5E). Antigenic determinants may fail to react

with the appropriate McAb if they have been changed, denatured, or extracted by the procedures used in sample preparation, gel electrophoresis, or transfer (Bers and Garfin, 1985). In the present study, alcohols were the common reagent in all of these procedures. Therefore, it is possible that the determinant recognized by McAb B3 may differ markedly from the other determinants in its sensitivity to alcohol. Alternatively, the determinant for McAb B3 may be of such low frequency that even short exposure to the alcohols completely denatured or extracted it.

The slight changes in the Western blots that were observed after *E. tenella* sporozoite proteins were digested with N-glycanase F are difficult to interpret. Prominent bands were still detected at 23 and 45 kDa in the digested samples (Fig. 6, lanes 2, 4, 6, 8), although they were less intense than those in the nondigested samples (Fig. 6, lanes 1, 3, 5, 7). These antigens apparently contained little or no N-linked carbohydrate, or the associated carbohydrate was refractory to the glycanase. However, with 2 of the McAb, 1A12 and 4115, an additional band (MW = ~18 kDa) was observed in the digested samples (Fig. 6, lanes 2, 4) that either was not in the nondigested samples (Fig. 6, lanes 1, 3) or was present in undetectable amounts. In addition, the bands having MW lower than 23 kDa appeared to be stronger after digestion. These changes may represent shifts in MW of antigens that prior to digestion migrated as part of the 23-, 45-, or 90-kDa bands and, after removal of carbohydrate by incubation in the glycanase, migrated as lower MW molecules. With 2 other McAb, 53A8 and 45C7, several bands (MW ~20 kDa) were detected in the nondigested samples (Fig. 6, lanes 5, 7) but not in the digested samples (Fig. 6, lanes 6, 8).

The RB remains an enigma. The structures, which occupy a large proportion of the sporozoite mass, undergo marked changes after the sporozoite invades a cell; among the species of *Eimeria* that infect poultry, these changes include the merging of anterior and posterior RB, the release of material from both the anterior and posterior RB, and the splitting of the RB into several smaller bodies that decrease in size and eventually vanish (Fayer, 1969). It has been suggested by a number of investigators that the RB plays a role during early development of the parasite. The avian *Eimeria*, although they in-

vade foreign hosts, will develop only in the specific host. This behavior suggests that interactions between specific parasite and host cell components must occur before development can proceed. The RB antigens that were shown in this study to have limited distribution among the avian *Eimeria* may be involved in these interactions.

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## LYSATES OF *SARCOCYSTIS CRUZI* BRADYZOITES STIMULATE RAW 264.7 MACROPHAGES TO PRODUCE TUMOR NECROSIS FACTOR (CACHECTIN)

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**ABSTRACT:** Lysate of *Sarcocystis cruzi* bradyzoites induced RAW 264.7 macrophages to release a factor that killed L 929 cells. Rabbit antibody to murine tumor necrosis factor (TNF) inhibited this killing effect, indicating that the factor causing cell death was similar to TNF (also called cachectin). Based on the present findings we hypothesize that TNF mediates, at least in part, the disease associated with this parasitic infection.

Lysates of asexual stages of species of *Sarcocystis*, *Toxoplasma*, and *Eimeria* are toxic when injected into various mammalian hosts (M'Gowan, 1914; Lunde and Jacobs, 1964). The substances imparting toxicity have not been chemically defined and their mechanism of action has not been determined. Recently, a lysate of *Sarcocystis cruzi* bradyzoites was shown to possess lectin activity separable from toxic activity (unpubl.). The lysate produced a reaction in rabbits similar to that produced by bacterial endotoxin. Bacterial endotoxin also has the ability to stimulate macrophages to produce the monokine called cachectin or tumor necrosis factor (TNF) (Beutler and Cerami, 1987). TNF has been implicated as a cause of a profound wasting diathesis in rabbits with trypanosomiasis (Beutler and Cerami, 1987). The present studies were therefore undertaken to determine if lysate from *S. cruzi* bradyzoites also possessed the ability to stimulate TNF production by macrophages. If so, such findings might provide at least a partial explanation for the underlying mechanism resulting in the profound wasting associated with sarcocystosis (Fayer and Dubey, 1986).

### MATERIALS AND METHODS

#### Lysate preparation

Bradyzoites of *S. cruzi* were obtained, purified, and used to prepare the lysate (Gasbarre et al., 1984). Two batches of lysate were each prepared using bradyzoites from 2 different experimentally infected calves. Batch 2 lysate differed from batch 1 in its preparation by the use of sterilized utensils to eliminate endotoxin contamination. Basically, bradyzoites were disrupted by alternate freezing and thawing followed by mechanical disruption with a Polytron homogenizer (Brinkmann Instruments, Rexdale, Ontario, Canada). Particulate matter was removed by centrifugation and the lysate

was dialyzed to retain products greater than 6,000-8,000 MW. Lysate was filtered through a sterile 0.22- $\mu$ m filter and stored at  $-70^{\circ}\text{C}$  in 2 ml aliquots. Several drops of lysate were placed in nutrient broth and incubated at  $37^{\circ}\text{C}$  for 5 days to test for bacterial contamination. Possible endotoxin contamination was determined by *Limulus* amoebocyte lysate (LAL) assay test kits (Sigma, St. Louis, Missouri). The protein content was determined by BioRad protein assay kit (Bradford, 1976). Portions of batch 1 lysate were adsorbed with Sepharose 4B and Polymyxin B-Sepharose. Five ml of lysate were adsorbed with 5 ml of Sepharose 4B in 40 ml of saline for 18 hr with stirring. The slurry was centrifuged at 150 g for 10 min; then the supernatant was removed and concentrated on a Minicon concentrator (Amicon Corp., Danvers, Massachusetts) to the original volume. Polymyxin B-Sepharose adsorption was performed in a similar manner using 100 mg of dehydrated gel in a final volume of 100 ml.

#### TNF bioassay

Based on previous studies indicating that RAW 264.7 murine macrophages produce TNF in response to stimulation by lipopolysaccharide from *Escherichia coli* (Mahoney et al., 1985) and that mouse L cells were highly susceptible to killing by TNF (Carswell et al., 1975), an *in vitro* assay for production and detection of TNF was established. Stock cultures of RAW 264.7 macrophages (T1B 71) and L 929 cells (CCL 1) were purchased from the American Type Culture Collection (Rockville, Maryland) and grown in a mixture of RPMI-1640 (GIBCO, Grand Island, New York) with 2 mM glutamine, 1 mM sodium pyruvate, and antibiotics (50 IU penicillin/ml, 50  $\mu$ g streptomycin/ml) as well as 10% fetal bovine serum (FBS) in T-25 flasks (Costar, Cambridge, Massachusetts) at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$ :95% air atmosphere.

The assay was conducted in the following manner. RAW cells were plated at  $1.5 \times 10^5$  cells/well in each well of a 24-well tissue culture plate (Costar), and L 929 cells were plated at  $5 \times 10^4$  cells/well in each well of a 24-well tissue culture plate with culture medium as above. After 24 hr, culture medium on RAW cells was replaced with serum-free medium containing lysate or other products. Each treatment was tested in triplicate. Wells with untreated cells were used as controls for each assay. These cells also served as an indicator for the possible contamination of serum with

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endotoxin. If present, the endotoxin should stimulate the production of TNF, which would affect the L 929 cells. On day 3, 24 hr after treatment products were added to RAW cells, the medium was removed from each well of RAW cells and filtered through 0.22- $\mu$ m filters (Millex-GV, Millipore). Culture medium was then removed from the L 929 cells and replaced with 0.30 ml of the RAW medium supplemented with 0.10 ml of RPMI-1640 and FBS to make a final concentration of 10% FBS. Possible direct effects on L 929 cells of treatment products carried over from the RAW cell medium were tested by adding treatment products to medium from untreated RAW cells and exposing L 929 cells to the mixture. The treated L 929 cells were incubated for 24 or 48 hr and observed microscopically on day 4 (and on day 5 for 48 hr incubation). The proportion of viable cells was determined by Coulter Counter counts of adherent cells. First, each well was washed twice with calcium-free and magnesium-free Hanks' balanced saline solution (HBSS). Then adherent cells were removed with a solution of 0.5% trypsin and 0.2% EDTA in HBSS. The reaction was stopped by addition of 2 volumes of cold RPMI-1640 with 10% FBS when microscopic observations revealed that cell detachment was complete. The well contents were dispersed and added to a Coulter Counter vial of balanced electrolyte solution (Isoton II, Curtin-Matheson Scientific, Inc., Houston, Texas) and counted by Coulter Counter (Model ZBI, Coulter Electronics, Inc., Hialeah, Florida). Percent viability of cells in treatment wells was calculated in relation to the number of cells in control wells.

To ensure that Coulter Counter counts of adherent L 929 cells were an accurate representation of viable cells and that nonadherent cells were in fact nonviable, viability was determined by trypan blue dye exclusion as previously demonstrated by Hass et al. (1985). L 929 cells were exposed for 24 and 48 hr to culture media from lysate-exposed RAW cells. Other L 929 cells were exposed for 24 and 48 hr to culture medium containing *Escherichia coli* bacterial endotoxin (5  $\mu$ g/well). Still other L 929 cells remained as untreated controls. Four replications were tested. The nonadherent cells floating in the culture medium and trypsin-released adherent cells in suspension were added to an equal volume of a 0.5% solution of trypan blue dye. Viable and nonviable cells as determined by dye exclusion were enumerated by hemacytometer counts.

#### Experimental design

Three experiments were conducted using batch 1 lysate. In experiment 1, triplicate wells of L 929 cells were enumerated at 24 and 48 hr after incubation with media from variously stimulated RAW cells to determine the best time for enumeration in subsequent experiments. L 929 cells were incubated in medium from unstimulated RAW cells and from RAW cells treated with either 5  $\mu$ g *E. coli* endotoxin, 100  $\mu$ l of *S. cruzi* lysate, heat-treated (65 C for 30 min) *S. cruzi* lysate, *S. cruzi* lysate adsorbed with Sepharose 4B, or *S. cruzi* lysate adsorbed with Polymyxin B-Sepharose. Sepharose 4B was used to adsorb lectin from the lysate (unpubl.) and Polymyxin B-Sepharose was used to remove any contaminating bacterial endotoxin.

In experiments 2 and 3 adherent L 929 cell counts

were determined at 48 hr only, and tests on endotoxin and lysate were repeated as in experiment 1 with the addition of rabbit antimurine recombinant TNF (R anti-m TNF) (Genentech, South San Francisco, California) to an additional set of wells for each test condition. R anti-m TNF had an activity of  $6.4 \times 10^5$  neutralizing units/ml and was used at 1/200 dilution as recommended by the producer.

To confirm specific activity of the *S. cruzi* bradyzoite lysate, TNF assays were repeated with batch 2 of the lysate, which was carefully prepared with glassware and solutions treated and prepared to be endotoxin-free and sterile. This extract was not treated with Polymyxin B or Sepharose 4B, and it was tested at 50  $\mu$ l/well. In experiments 4 and 5 triplicate wells of L 929 cells were enumerated 48 hr after incubation with media from RAW cells stimulated by the addition of batch 2 lysate or endotoxin, or after incubation with media from untreated RAW cells. In experiment 4, 1 set of L 929 cells received media from lysate-stimulated RAW cells to which R anti-m TNF was added.

Data were analyzed by analysis of variance, and means were compared with Duncan's multiple range test. Means were considered significantly different at  $P < 0.05$  (Freund and Little, 1981).

#### RESULTS

Bacterial contamination was not detected after incubation of lysates in nutrient broth for 5 days. Protein content of batch 1 of the lysate was 1,700 mg/ml. Batch 2 contained 2,700 mg protein/ml.

The LAL assay for endotoxin indicated that batch 1 had 10–16 ng endotoxin activity/ml of untreated lysate. After Polymyxin B adsorption, endotoxin activity was 1–4 ng/ml but was 64 ng/ml after Sepharose 4B treatment. The LAL assay for batch 2 indicated an endotoxin activity of 0.5 ng/ml. Endotoxin activity on cells treated with lysates was one-tenth of these levels for batch 1 and one-twentieth of the level for batch 2 due to dilution with culture medium.

Tests for cell viability by trypan blue exclusion indicated in all 4 replications that at 48 hr after exposure to culture medium from lysate or endotoxin-treated RAW cells over 50% of the L 929 cells were nonadherent and 100% of the nonadherent cells were nonviable; of the remaining adherent cells in the same wells 0–55% were nonviable. In comparison, of L 929 cells exposed to medium from nontreated RAW control cultures less than 0.001% of the L 929 cells were nonadherent and nearly all of these were nonviable; less than 0.01% of the adherent cells were nonviable.

In experiment 1 the reduction in number of adherent L 929 cells was much greater at 48 hr incubation with culture medium from RAW-treated cells than at 24 hr incubation with similar

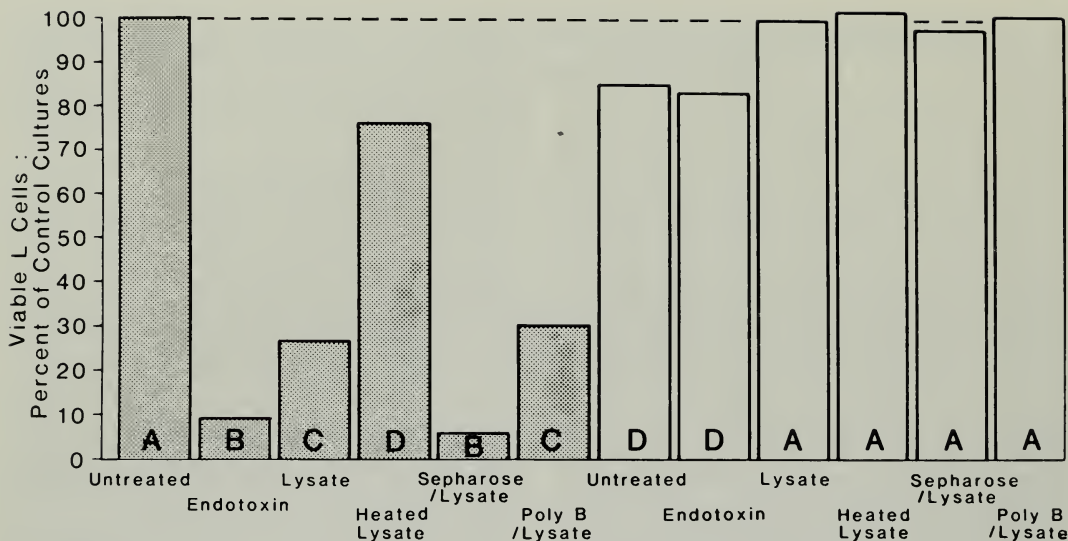


FIGURE 1. Experiment 1. Effects of treatments on L cell survival 48 hr after incubation with various test reagents. Solid bars represent L cell cultures exposed to medium from variously treated RAW cells. Open bars represent L cell cultures directly exposed to those reagents with no culture medium from RAW cells. Bars with different letters are significantly different ( $P \leq 0.05$ ).

culture medium (Fig. 1). Therefore, subsequent experiments were conducted with 48-hr incubation times. Culture media from RAW cells treated with endotoxin, untreated *S. cruzi* lysate, heat-treated lysate, Sephadex 4B-adsorbed lysate, and Polymyxin B-Sephadex-adsorbed lysate significantly reduced the number of viable L 929 cells compared with those incubated in medium from untreated RAW cells (Fig. 1). Direct exposure of L 929 cells to untreated *S. cruzi* lysate, heat-treated lysate, Sephadex-adsorbed lysate, and Polymyxin B-Sephadex-adsorbed lysate did not significantly affect viability of L 929 cells as compared with those that received untreated culture medium from RAW macrophage cultures. Nor was there a significant difference in L 929 cell numbers between untreated L cells and those treated directly with endotoxin.

Results of experiments 2 and 3 were virtually identical (Fig. 2). L 929 cells incubated with culture medium from RAW cells treated with endotoxin or *S. cruzi* lysate were significantly reduced in number compared with controls incubated with culture medium from untreated RAW cells (Fig. 2). Addition of R anti-m TNF to each of the culture media from treated RAW cells resulted in a marked neutralization of the killing effect on L 929 cells as indicated by significantly greater numbers of L cells in the pair-matched cultures

(Fig. 2). Addition of R anti-m TNF directly to L 929 cell cultures did not affect viability.

Results of experiments 4 and 5 indicate that L 929 cells incubated for 48 hr with media from lysate-stimulated RAW cells contained 15% and 39% of the number of cells found in cultures incubated with media from nontreated RAW

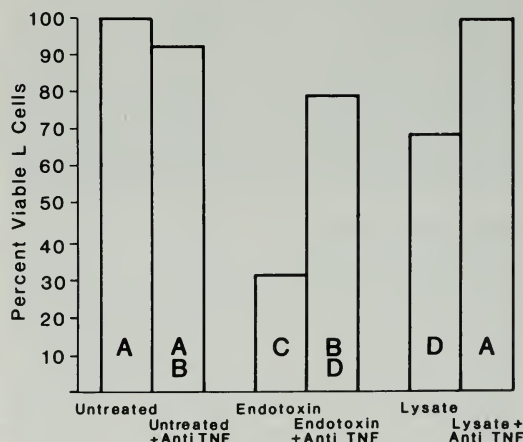


FIGURE 2. Experiments 2 and 3 combined to show percent viable L cells at 48 hr after incubation with culture medium from RAW cells. Culture medium containing rabbit antibody to cloned murine TNF is designated as +anti TNF. Bars with different letters are significantly different ( $P \leq 0.05$ ).



cells, respectively. Addition of R anti-m TNF to media from RAW cells in experiment 4 resulted in 100% protection from the killing effect of the media. Cultures of L 929 cells incubated with media from endotoxin-stimulated RAW cells contained 10% and 19% of the number of cells in cultures incubated with media from nontreated RAW cells in experiments 4 and 5, respectively.

## DISCUSSION

Results indicate that lysates of *S. cruzi* bradyzoites induced RAW macrophages to release a factor into the culture medium that killed L 929 cells. Untreated lysate, heat-treated lysate, Sepharose-adsorbed lysate, Polymyxin B-Sepharose-adsorbed lysate, and bacterial endotoxin all stimulated release of the factor. When rabbit antibody to cloned murine TNF was added to the culture medium from macrophages stimulated by either lysate or endotoxin, the killing effect on L cells was inhibited significantly, indicating that the factor responsible for the killing was antigenically similar to, if not identical to, murine TNF- $\alpha$ . The finding that *S. cruzi* lysate adsorbed with either Sepharose 4B or Polymyxin B-Sepharose still induced the RAW macrophages to release the TNF-like factor suggests that neither the galactose-specific lectin previously identified in the lysate (unpubl.) nor a bacterial endotoxin contaminant were responsible for stimulating the RAW cells. Preparation of batch 2 lysate with special care to avoid endotoxin contamination and testing to find endotoxin levels of only 0.025 ng/well substantiate the conclusion that some substance from the lysate of the *Sarcocystis* bradyzoites, and not endotoxin contamination, was the stimulus for RAW macrophages.

Based on the results obtained in the present study it is important to examine the pathologic effects of *Sarcocystis* *in vivo* and speculate on a possible mechanism of disease. It has been observed that acute sarcocystosis resembles endotoxic shock characterized by fever, hemorrhage, edema, and possibly death, with a histologic presentation of perivascular cuffing containing many macrophages and lymphocytes (Fayer and Dubey, 1986). Chronic sarcocystosis is characterized by cachexia, muscle atrophy, stunted growth, and has a similar inflammatory cell component (Fayer and Dubey, 1986). Because administration of TNF to animals has resulted in signs identical to endotoxemia including fever, coagulo-

pathy, and death, and smaller doses of TNF have resulted in anorexia, weight loss, and cachexia (Beutler and Cerami, 1987), it is hypothesized that TNF might be at least one of the mediators responsible for the pathogenesis of sarcocystosis. Metabolic perturbations in cattle with experimentally induced sarcocystosis revealed a depression in plasma concentration of somatomedin C with a concurrent elevation in somatostatin that was significant beyond the effects of nutritional status (Elsasser et al., 1986). The possibility that TNF might influence growth directly by inhibiting bone growth (Bertolini et al., 1986) or indirectly by affecting growth regulators such as somatomedin or somatostatin offers an approach toward better understanding the molecular mechanisms responsible for poor growth and poor feed utilization due to parasitism.

Lysis of L 929 cells by TNF is an established bioassay for biological activity of TNF (Hass et al., 1985). RAW 264.7 cells have been shown to secrete a substance with activity of cachectin-TNF (Mahoney et al., 1985). Combining the 2 *in vitro* techniques into the test system used in this study may have application for the testing of other biological products or whole organisms for their ability to stimulate TNF production.

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## TRICHINELLA SPIRALIS INFECTIONS OF INBRED MICE: GENETICS OF THE HOST RESPONSE FOLLOWING INFECTION WITH DIFFERENT TRICHINELLA ISOLATES

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**ABSTRACT:** The immune response of inbred strains of mice was studied following infection with isolates of *Trichinella* from a pig (P<sub>1</sub>), an arctic fox (AF<sub>1</sub>), and *T. spiralis* var. *pseudospiralis* (TP). Strains of mice previously characterized as highly resistant to a separate pig isolate of *T. spiralis* responded to the P<sub>1</sub> and AF<sub>1</sub> isolates by expelling over 80% of the worms by day 10 postinfection (PI), and by suppressing the *in vitro* release of newborn larvae by female worms. However, the response induced by AF<sub>1</sub> worms was expressed more quickly when compared to responses induced by the P<sub>1</sub> and TP isolates. The host response to TP was less as recovery was always higher at day 10 PI and antifecundity effects were not induced in TP worms even in highly resistant strains of mice. Strains of mice previously characterized as susceptible to *T. spiralis* infection were slow to develop resistance when compared to the resistant mouse strains, but even among the susceptible strains, infection with AF<sub>1</sub> induced a more rapid response. The mouse strains used in these experiments allowed us to assess the role of the major histocompatibility complex (MHC) and/or non-MHC genes in influencing the responses observed. As previously reported for a pig isolate of *T. spiralis*, both MHC and non-MHC genes influenced the rate at which worms were expelled from the gut and the host response that limits the fecundity of adult female worms.

There is ample evidence that inbred strains of mice respond differently to strains or isolates of *Trichinella spiralis* (Wassom et al., 1983; Wakelin, 1985). These responses include differences in the rate at which adult worms are expelled from the intestine, increased or decreased larval production by female worms (measured under *in vitro* conditions), numbers of larvae recovered from the muscle of infected mice, and a variety of immunological parameters (cellular and humoral responses). Although most of these host-parasite interactions focused on differences in the host response to the parasite, there is an increasing awareness that parasite genetics, i.e., phenotypic traits such as infectivity and immunogenicity, play an important role in the host-parasite association. The biological variability of *Trichinella* has been reviewed by Dick (1983), and it was evident that strains of *Trichinella* with differing characteristics occur frequently, particularly under natural conditions. Differences in the host response to different strains or varieties of *Trichinella* are known (Dick, 1983), but not much is known about the response of inbred

strains of mice to *Trichinella*. Palmas et al. (1985) evaluated the immune response of inbred strains of mice to *Trichinella spiralis* and *T. pseudospiralis* and concluded that they were very similar. Almond et al. (1986) found that adults and larvae of *T. spiralis* and *T. pseudospiralis* displayed a mixture of common and species-specific proteins. Wassom et al. (1988), employing challenge infections and using an antigen-specific lymphocyte proliferation assay, demonstrated that different isolates of *Trichinella* showed qualitative and/or quantitative differences in the expression of functionally relevant antigens. These differences were reflected in the rates at which worms were expelled from the gut, and in the ability of the host to limit the production of newborn larvae.

The objective of this study was to assess the immune responses of inbred strains of mice to different isolates of *Trichinella*.

### MATERIALS AND METHODS

#### Strains of parasites and mice

The strains of *Trichinella* used in the study are designated as follows: P<sub>1</sub> (an isolate of *T. spiralis* from a pig [designated as pig; 43°00'N, 81°00'W; 1953]); TP (*T. spiralis* var. *pseudospiralis* [obtained from G. Faubert, MacDonald College, McGill University]); AF<sub>1</sub> (an isolate from an arctic fox [designated as AF<sub>1</sub>; 69°15'N, 105°00'W; 1980]). The 3 strains of *Trichinella* have been shown to differ in their infectivity for outbred strains of mice (Bober and Dick, 1983; Dick, 1983). Prior to the present study all strains of the parasite were passaged through CRL COBS CFW outbred Swiss

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TABLE I. Susceptibility of inbred mouse strains to infection with *T. spiralis*.\*

Strain	H-Z haplotype	Percent of C3H control	Resistance phenotype
C3HeB/FeJ	k	100	Susceptible
B10.BR	k	104	Susceptible
AKR/J	k	62	Resistant
B10.Q	q	47	Resistant
SWR/J	q	45	Resistant

\* From Wassom et al. (1983).

Webster mice. For the present study all strains of *Trichinella* were passaged and maintained in C3HeB/FeJ mice prior to experimental infection.

The strains of mice used in these studies were chosen as they either possessed common genetic backgrounds but different alleles at genes within the major histocompatibility complex (MHC), or shared common MHC genes but expressed different genetic backgrounds. This allowed us to independently observe the influence of MHC or non-MHC genes on the responses observed. The strains of mice used in these experiments, and data relating to their susceptibility to *T. spiralis* as reported by Wassom et al. (1983) are shown in Table I.

#### Mouse maintenance

All mice used in the experiment were 5-8-wk-old males reared in the immunogenetics mouse colony at Cornell University. Mice were housed 5/cage in 29.5 × 18.5 × 13-cm polycarbonate cages, fed Charles River RMH 1000 formula *ad libitum*, and were on a 12-hr photoperiod.

#### Infections

Muscle larvae for infections with each isolate of *Trichinella* were obtained from carcasses of freshly killed C3HeB/FeJ male mice. Mice were killed by cervical dislocation, skinned, eviscerated, and blended individually in a Waring blender (100 ml of 1% HCl-pepsin solution, P7000 Sigma Chemical Co., St. Louis, Missouri). The blended material was incubated with shaking at 37 C for 2 hr in an additional 100 ml of 1% HCl-pepsin (Orbit Environmental Shaker Labline Inc.). After digestion, worms were separated from the digest by passing the incubated material over a 100-mesh sieve (5-in. diameter) and collecting the larvae on a 250-mesh sieve stacked below it. Larvae were washed from the 250-mesh sieve into a 50-ml conical centrifuge tube using 0.85% saline, and washed 5 times by resuspending the larvae in saline, and allowing them to settle. All experimental mice were infected per os with 150-200 muscle larvae using a 0.5-ml syringe equipped with a blunt, curved 18-gauge needle.

Adult worms were recovered from the intestines of mice killed by cervical dislocation. The entire intestine was removed to a 100-ml plastic petri dish containing 25 ml of warm phosphate-buffered saline (PBS), pH 7.2. The small intestine was split longitudinally along its entire length and incubated at 37 C for at least 4 hr. The intestine was then discarded and the worms that migrated out of the tissues and into the saline were counted using a dissecting microscope. Each experi-

ment utilized 5 mice of each strain at each sampling time (days 6, 10, 14 PI).

#### *In vitro* release of larvae by female worms

Female worms were recovered on days 6, 10, and 14 PI from the intestines of mice and placed in petri dishes containing PBS at 37 C for at least 2 hr. A sample of 8-10 worms from each of 5 experimental mice for each sample time was collected whenever possible for *in vitro* studies. Female worms were placed individually in wells of a 96-well microtitre plate in 200 µl of culture medium. The medium consisted of RPMI-1640, 29% newborn calf serum, and a 1% antibiotic-mycotic solution (GIBCO, Grand Island, New York). Plates were incubated at 37 C, 5% CO<sub>2</sub> for 24 hr. Newborn larvae produced were counted with the aid of a Biostar inverted microscope.

#### RESULTS

Figure 1 shows data comparing adult worm counts on day 10 postinfection (PI) for 5 strains of mice infected separately with the P<sub>1</sub>, AF<sub>1</sub>, and TP isolates of *Trichinella*. Three of the mouse strains share the H-2<sup>k</sup> haplotype (B10.BR, C3HeB/FeJ, and AKR/J) and 2 strains share H-2<sup>q</sup> alleles (B10.Q and SWR/J). Figure 2 shows counts for newborn larvae produced by female worms harvested from these mice. Results for both figures are expressed as a percentage of the mean count from the appropriate day-6 PI controls; on day 6 of a primary infection, the immunologically mediated expulsion of worms from the gut has not yet begun, and antifecundity responses are likewise not yet evident (Wassom et al., 1984). Although worm counts and fecundity assays were performed on day 14 PI, these data were not included in the figures as few or no worms were found in many of the host-strain/parasite-isolate combinations.

It is clear from these data that infections with AF<sub>1</sub> are expelled quickly by most strains of mice when compared to infections with P<sub>1</sub> or TP. For example, B10.Q mice did not expel significant numbers of P<sub>1</sub> or TP worms by day 10 PI, whereas approximately 50% of the AF<sub>1</sub> worms had been expelled by this time. Although AKR/J mice were quick to expel all infections compared to B10.BR or C3HeB/FeJ mice, AKR/J mice expelled proportionally more AF<sub>1</sub> worms (approximately 80%) than they expelled infections with other isolates (approximately 50% of P<sub>1</sub> and 40% of TP). SWR/J mice expelled a large proportion of worms of all 3 isolates of *Trichinella* by day 10 PI, but the AF<sub>1</sub> worms were completely expelled by day 10 PI, a time when some P<sub>1</sub> and TP worms were still present.



The ability of inbred strains of mice to limit the fecundity of adult female worms of the  $P_1$ ,  $AF_1$ , and TP isolates tended to parallel the results observed for the rates at which these parasites were expelled from the gut. However, it is clear that the antiadult and the antifecundity responses have different kinetics (Fig. 2). For example, the  $P_1$  and  $AF_1$  isolates induced almost identical antifecundity responses in the respective panel of hosts in which they were tested. In contrast, the TP isolate failed to induce an effective antifecundity response in any of the mouse strains, even those shown to respond strongly to other isolates. In each group of mice infected with TP, more newborn larvae were produced on day 10 than on day 6, and when sufficient worms were available for testing on day 14 PI, fecundity of TP was not reduced beyond levels observed on day 6 PI (data now shown).

#### DISCUSSION

In the present study we used well-defined strains of *Trichinella* to infect a wide range of inbred strains of mice. Our results support the observations of Wassom et al. (1983) who reported that both non-H-2 genes and H-2 genes influence levels of susceptibility to *Trichinella* infections of mice. It was clear from our data that differences in immunogenicity among strains of parasites could be overlooked if only strong responder strains of mice are tested. For example, SWR/J mice expelled up to 80% of worms of all 3 isolates prior to day 10 PI (Fig. 1). However, when *in vitro* release of newborn larvae are assessed (Fig. 2) it was evident that  $P_1$  and  $AF_1$  induced a stronger antifecundity response than TP regardless of the strength of the expulsion response.

In the present study the genetic background of the mouse strains tested had a strong influence on the results observed. SWR/J expelled proportionally more adult worms and suppressed larval release to a greater extent than did B10.Q mice even though they share the H-2<sup>q</sup> haplotype. In addition, B10.BR, C3HeB/FeJ, and AKR mice (all H-2<sup>k</sup>), also differed in their responses to the 3 *Trichinella* isolates; AKR/J mice were considerably more resistant than were the other 2 strains. Mice sharing a common C57BL/10 genetic back-

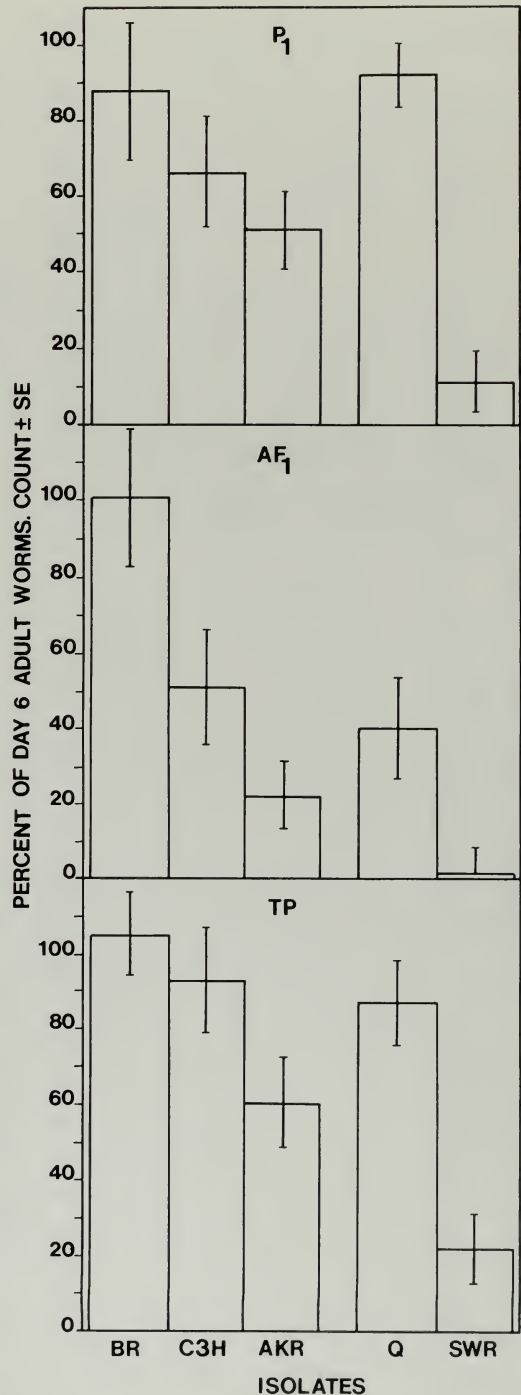
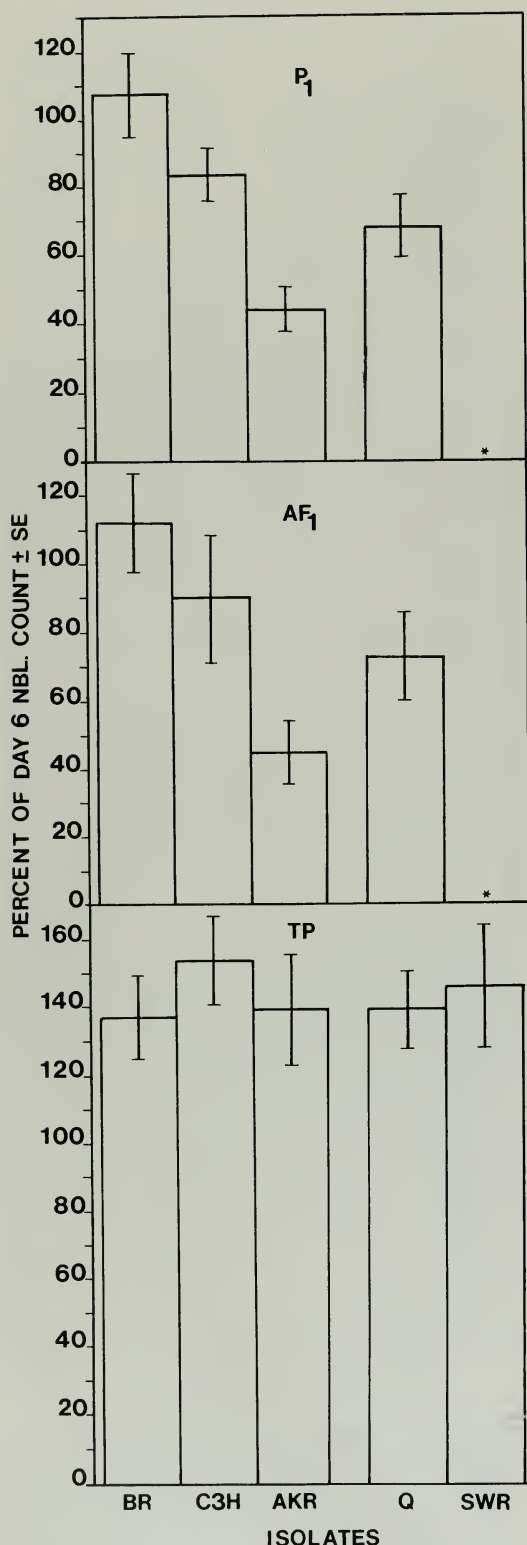


FIGURE 1. Adult worm recoveries from inbred mouse strains infected with the  $P_1$ ,  $AF_1$ , and TP isolates

of *Trichinella*. Results are expressed as the percentages ( $\pm$ SE) of worms remaining on day 10 PI when compared to counts for day-6 controls. Mouse strain designations are shown within the vertical bars and are abbreviated as follows: BR (B10.BR [H-2<sup>k</sup>]); C3H (C3HeB/FeJ [H-2<sup>k</sup>]); AKR (AKR/J [H-2<sup>k</sup>]); Q (B10.Q [H-2<sup>q</sup>]); SWR (SWR/J [H-2<sup>q</sup>]).



ground but expressing different H-2 alleles gave predictable results. B10.BR and B10.Q mice rejected about the same proportions of  $P_1$  and TP through day 10 PI, but B10.Q rejected a higher proportion of  $AF_1$  than did B10.BR mice. B10.Q mice suppressed the *in vitro* larval release of both  $P_1$  and  $AF_1$  worms by about 30%. This suggests that although the  $P_1$  and  $AF_1$  isolates are similar in their ability to stimulate functional antifecondity responses, they are quite different in stimulating the expulsion response.

Our observations clearly indicate that the 3 isolates differ in their ability to induce functional immunity in the host. That TP differed from  $AF_1$  and  $P_1$  was expected as there is ample evidence that it differs from all other isolates of *Trichinella* (Palmas et al., 1985; Almond et al., 1986; Wassom et al., 1988). However, the lack of an antifecondity response by day 10 PI and indeed as late as day 14 PI was unexpected. The lack of a strong antifecondity response to TP may relate to fewer larvae being produced by females at day 6 PI; for example, female worms recovered from B10.Q mice produced a mean of 25 larvae at day 6 PI, 30 larvae at day 10 PI, and 35 larvae at day 14 PI. This compares to a mean of 33 larvae for  $AF_1$  and a mean of 52 larvae for  $P_1$  females at day 6 PI. It might be argued that among the isolates of *Trichinella* tested, differences in size or number of worms establishing in the intestine, or reduced larval production by female worms could result in more or less antigen being available to stimulate an immune response. Such dose-related considerations merit additional experimentation. However, worm size or numbers alone would not fully explain the results observed here as the kinetics of immune responsiveness to different *Trichinella* suggests differences among the isolates in the expression of functionally relevant antigens (Wassom et al., 1988).

#### ACKNOWLEDGMENTS

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FIGURE 2. Newborn larval production *in vitro* by  $P_1$ ,  $AF_1$ , and TP female worms harvested from inbred mouse strains. Results are expressed as the percentage ( $\pm$ SE) of newborn larvae produced by female worms on day 10 PI when compared to counts for day-6 controls. Abbreviations for mouse strains tested are as specified in Figure 1. An \* in place of a vertical bar indicates that insufficient female worms were available on day 10 to perform the *in vitro* fecundity assay.

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## GROWTH AND DEVELOPMENT OF WINTER TICK, *DERMACENTOR ALBIPICTUS*, ON MOOSE, *ALCES ALCES*

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**ABSTRACT:** Moose, *Alces alces*, were infested with 21,000 or 42,000 larval *Dermacentor albipictus* at the end of September. Larvae grew rapidly and molted to the nymphal stage 10–22 days after infestation. The nymphal stage lasted approximately 3 mo until mid-January and was characterized by a diapause. The diapause is likely an adaptation to survival in cold climates. Nymphs started engorging in January and adults were seen with increasing abundance from mid-January to March and April. The minimum parasitic period was 175 days. Growth of larvae and nymphs was similar on moose given different numbers of larvae and was generally similar between a moose infested in November and moose infested earlier. Dimensions and stages of development throughout the parasitic phase are given. Game enforcement officers are encouraged to use these data for determination of season of death of moose.

The winter tick, *Dermacentor albipictus* (Packard, 1869), was originally described from moose, *Alces alces*. However, much of our knowledge of the development of *D. albipictus* is based on studies of ticks from cattle and horses (Bishopp and Wood, 1913; Howell, 1939a, 1939b; Drummond, 1967; Osburn, 1981) and on the *nigrolin-eatus* form of the species that was synonymized with *D. albipictus* by Cooley (1938). Field observations of winter ticks associated with hair loss or mortality of moose has increased interest in moose–winter tick relationships (Addison et al., 1979; Samuel and Barker, 1979; Glines and Samuel, 1984; McLaughlin and Addison, 1986). Winter ticks were present on wild moose from September to May in northern Ontario (Addison et al., 1979), and the timing of stages of development of *D. albipictus* on wild moose in Alberta has been documented (Samuel and Barker, 1979; Glines and Samuel, 1984).

The objectives of the present study are to (1) describe the growth and development of winter ticks on moose under controlled conditions, (2) determine if the number of larvae placed on moose influences growth and development of the ticks, (3) assess if the timing of infestation of moose affects patterns of growth and development of the ticks, and (4) to compare development of *D. albipictus* from captive moose in Ontario with development of *D. albipictus* on livestock and moose elsewhere.

### MATERIALS AND METHODS

Eighteen newborn moose were collected and raised in Algonquin Provincial Park, Ontario (45°33'N,

78°35'W) during the spring of 1982 (Addison et al., 1983). One male and 1 female were placed in each of 6 adjacent pens (29.6 × 16.5 m) and 4 males with 2 females were placed in another pen (29.6 × 35.0 m). All moose were fed *ad libitum* on a pelleted ration containing 16% crude protein, 2.5% crude fat, and 16% crude fibre. Fresh feed was supplied daily.

Larval *D. albipictus* were collected during September and October 1982 in Algonquin Park by dragging white flannelette sheets over vegetation in wild moose habitat. The larvae were evacuated (–40 KPa relative pressure) from sheets into 50-ml test tubes (approximately 5,000 per tube) that were sealed with fine mesh. Larvae were stored outside at ambient air temperature in a shaded location for 1–4 days before being applied at the base of the hair on the dorsal and lateral surfaces of moose. Moose were tethered on a short lead and fed browse during the application of larvae and for an additional 30 min to allow time for larvae to reach the hide without being removed by licking.

Infestation of 12 moose took place between September 17 and October 12 as larvae became available. Dosage rate ranged from 900 to 21,400 larvae per day, with each moose receiving 75% of its final tick load within 7 days of initial dose. The day on which most moose had accumulated 50% of their final tick load, 30 September, was considered the day of infestation. Six moose, H1 through H6, each received a high infestation of about 42,000 (41,612–42,007) larvae and 6 moose, M1 through M5 and M7, each received a relatively moderate infestation of about 21,000 (21,139–21,537) larvae. Another moose (M6) was infested with 19,000 larvae about a month later, on 6 November, in order to study the effect of time of infestation on rate of development of ticks. Five moose received no ticks. To control infestations acquired from the local environment, moose pens were sprayed with acaricide (Dursban M, Dow Chemical of Canada Ltd., Sarnia, Ontario N7T 7M1) 3 times at 2-wk intervals prior to the experiments in the spring of 1982.

Development of larvae was studied using ticks from 2 moose infested with 21,000 on 6 October and from M6 infested a month later. Approximately 25 larvae were collected every second day from day 2 to day 28 after infestation from each of the 3 moose.

Stages of development and nature of attachment of

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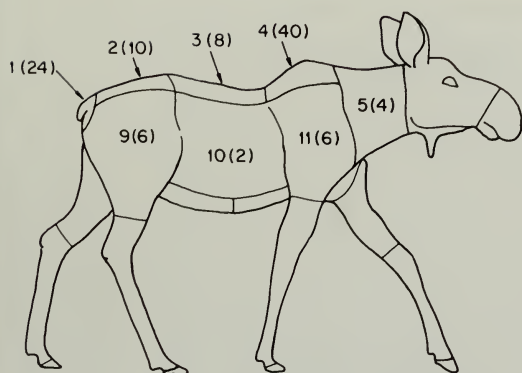


FIGURE 1. Location and number of *Dermacentor albipictus* removed monthly for growth studies. The number identifying the area is followed in parentheses by the number of ticks removed.

ticks found on transects on 8 moose were recorded monthly from October to March. Census plots were established on 8 regions of each moose (Fig. 1) by placing a permanent coloured mark on the hide of the moose in the centre of each region. Four transects ( $1 \times 10$  cm) were measured out every  $90^\circ$  about this point. Censuses of ticks were made on 21 October (21 days after infestation), 20–30 November (51–61 days), 15–20 December (76–81 days), 16–22 January (108–114 days), 21–24 February (144–147 days), and 10–13 March (161–164 days). The April census occurred when the 8 moose were killed 200–210 days after infestation. All ticks remaining on hides were classified by stage. Censuses between October and March sampled 762–1,290 ticks per moose. In the April census, 1,179–8,290 ticks per moose were sampled.

Growth of all stages was studied using a sample of 100 ticks removed monthly from areas 1–5 and 9–11 of each of the 13 infested moose (Fig. 1). Ticks were removed in equal numbers from right and left sides of the body and in proportion to the relative density of ticks found in the first census of transect plots. Ticks were not removed from the immediate vicinity of transects. Ticks were fixed in 70% ethanol. Prior to weighing, they were removed from the ethanol and classified by stage and relative engorgement. Surface fluids were allowed to evaporate. Each sample of 100 ticks was divided into 5 groups: larvae, unengorged nymphs (area  $< 2.31$  mm<sup>2</sup>), engorged nymphs (area  $\geq 2.31$  mm<sup>2</sup>), and adult males and females. Each group of ticks was weighed. Desiccation of ticks collected in March precluded accurate weighing. The total length, maximum width, scutal length, and scutal width of each tick, as defined by Cooley (1938), were drawn from a dorsal view using a compound microscope and drawing tube. Measurements of drawings were made with the aid of a stage micrometer and calipers.

Engorged females (480) that detached from moose on 8–10 April (190–192 days after infestation) were collected, washed to remove dirt, sponged with an absorbent towel, and weighed.

The dorsal surface area of ticks was calculated using the formula for calculating the surface area of an ellipse ( $A = \pi LW/4$ ) (see Davey et al., 1984). Graphs of growth

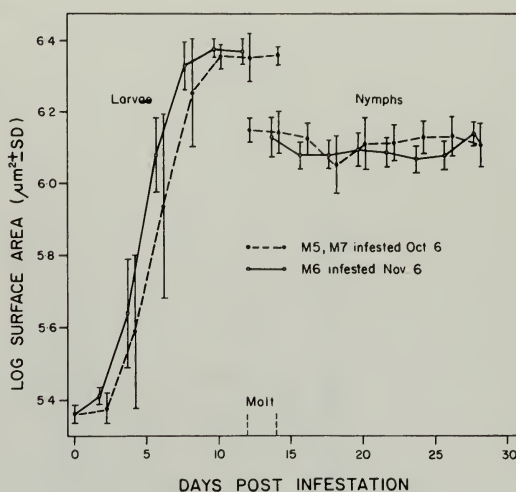


FIGURE 2. Growth of larval *Dermacentor albipictus* on moose. Area was calculated using the formula for the area of an ellipse.

were based on log transformations of the dorsal surface area. All other calculations used data that were not transformed. Analysis of variance (ANOVA) was used to compare the area of unengorged nymphs among months; the area of adults among months; and the weight of unengorged nymphs among months. When the ANOVA was significant, individual values were compared using the Newman-Keuls test. A nested ANOVA was used to compare the area of nymphs, the area of adults, and the weight of detached females among moose and infestation doses. The area of nymphs between moose infested in October with a moderate dose and the single moose infested in November was compared using ANOVA followed by Scheffé's test. The *t*-test was used to compare the area of male and female adult ticks, and to compare the weight of nymphs between infestation levels. The ratio of numbers of adults and nymphs were compared among moose with the *G*-test. All statistical tests followed procedures in Morrison (1982), Sokal and Rohlf (1981), and Zar (1984). Probabilities of  $< 0.05$  are considered significant.

## RESULTS

### Larvae

Larvae were  $0.60 \pm 0.026$  mm in length,  $0.49 \pm 0.02$  mm in maximum width, and  $0.23 \pm 0.01$  mm<sup>2</sup> in surface area, prior to attachment to moose. Following attachment, there were 3 distinct periods in the growth of larvae: an initial 2-day period of limited growth, a period of rapid growth through about 8–9 days after infestation, and a period of decreased growth prior to the molt (Fig. 2). Larvae from moose infested in October and November were similar in size 10 days after infestation (ANOVA,  $P = 0.09$ ) (Fig. 2). Twelve days after infestation, larvae were  $2.19 \pm$



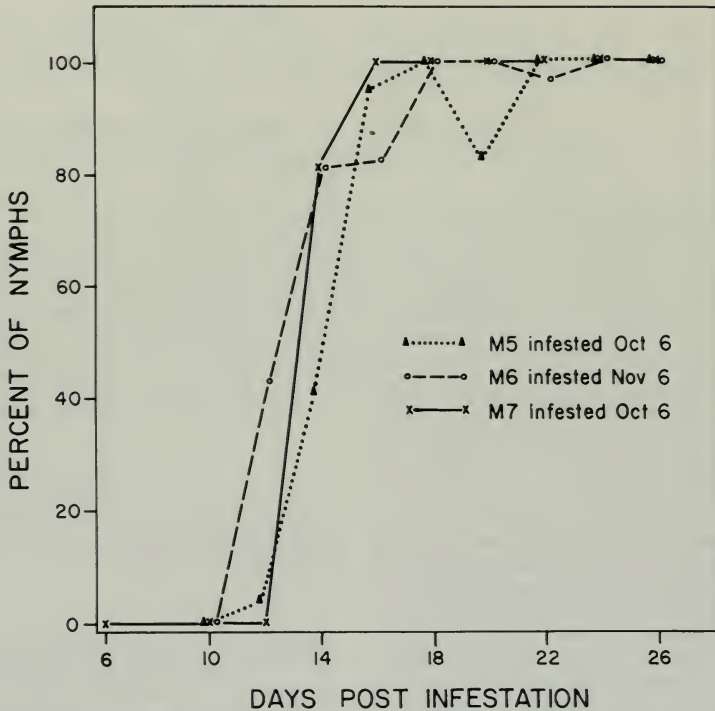


FIGURE 3. Timing of the larval molt to the nymphal stage by *Dermacentor albipictus* on moose.

0.18 mm long and  $1.32 \pm 0.12$  mm wide. Scuta were  $222 \pm 44$   $\mu$ m long and  $414 \pm 42$   $\mu$ m wide. At the molt, larvae were on average 9.5 times larger in area than at the time of attachment.

Larvae molted to the nymphal stage 10–22 days after infestation (Fig. 3). The duration of the larval stage was similar on 2 moose infested in October and on the moose (M6) infested 1 mo later (Fig. 3). At the first census of transects, 21 days after infestation, 97% of 1,015 ticks were nymphs. The proportion of nymphs on individual moose varied from 89 to 100%. No larvae were found during later censuses (Fig. 4).

**Nymphs**

Nymphs constituted 99.9 and 99.8% of more than 1,000 ticks found during each of the November and December censuses (Fig. 4). The few remaining ticks were adults. The proportion of ticks in the nymphal stage remained high in mid-January (101–108 days after infestation). All ticks on 5 moose ( $n = 592$ ) and all but 1 on an additional moose ( $n = 166$ ) were nymphs. On the remaining 2 moose, 89% and 94% of 65 and 47 ticks were nymphs. The proportion of nymphs decreased in subsequent censuses; very few were present in April (Fig. 4).

Newly emerged nymphs were considerably smaller than larvae measured shortly before the molt (Fig. 2; Table I). During the first 16 days following the molt, nymphs did not increase in size (Fig. 2) and were  $1.87 \pm 0.11$  mm long and  $0.88 \pm 0.09$  mm wide. Subsequently, there was a gradual increase in area (Fig. 5) such that in November unengorged nymphs were larger than in October on 9 of 11 moose (Newman-Keuls,  $P < 0.05$ ) (Table II). Similarly, unengorged nymphs in December were larger than in November on 8 of 11 moose (Newman-Keuls,  $P < 0.05$ ) (Table II). Growth slowed from day 75 (mid-December) to day 104 (early January) such that the area of unengorged nymphs was similar between these sampling periods on all 12 moose (Tables I, II). The weight of unengorged nymphs was similar from October to January (Newman-Keuls,  $P > 0.05$ ). In January, 4% of nymphs were engorged. The engorged nymphs were more than twice the length and width of unengorged nymphs.

In February, 60% of nymphs were engorged. Unengorged nymphs ( $n = 323$ ) were  $1.33 \pm 0.21$  mm<sup>2</sup> in area. They were smaller than unengorged nymphs in December and January and, on 6 of 12 moose, as small as or smaller than recently emerged nymphs in October (Newman-Keuls,



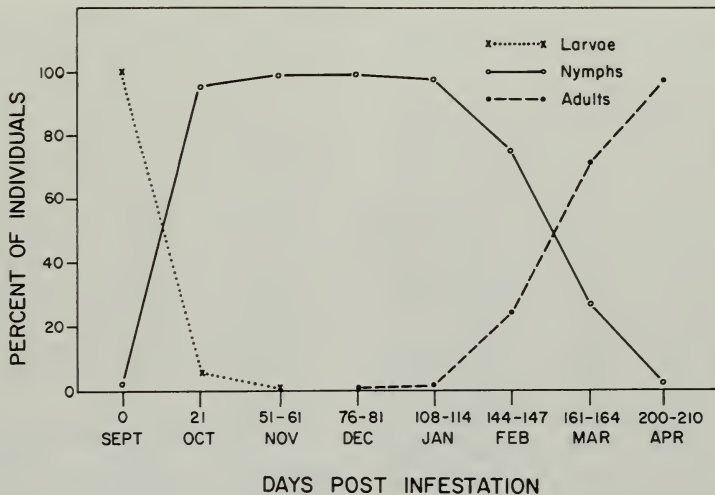


FIGURE 4. Timing of stages of development of *Dermacentor albipictus* on moose.

$P < 0.05$ ) (Table II). However, the unengorged nymphs weighed more in February ( $\bar{x} = 0.58$  mg) than in all previous months ( $\bar{x} = 0.49$ – $0.52$  mg) (Newman-Keuls,  $P < 0.05$ ). Engorged nymphs were larger in February than in January (Table I) (Newman-Keuls,  $P < 0.05$ ).

In March, unengorged nymphs averaged only  $1.09 \text{ mm}^2$  in area and were as small as or smaller than recently emerged nymphs in October (Newman-Keuls,  $P < 0.05$ ). Engorged nymphs were approximately 10 times larger than unengorged nymphs in February and March and also were larger than engorged nymphs in January and February (Table I).

#### Adults

Adults constituted 6% and 11% of the ticks examined on 2 moose in January, and were virtually absent from other moose. One of the moose with adults was from the moderately infested treatment group and the other from the heavily infested group. In January and February, 71% of adult ticks were males. The overall proportion of adult ticks on the moose increased from 1% in January to 24% in February (Fig. 4) with some variation among moose ( $G$ -test,  $P < 0.05$ ). For example, the moose with the highest proportion of adults in January also had 53% adults in February, compared to 16–29% ( $\bar{x} = 21.6$ ) adults on the other 7 moose. The proportion of adults continued to increase rapidly, reaching  $72 \pm 8.3\%$  of ticks by 161–164 days after infestation in March and 99% (95–100%) by 200–210 days after infestation in April (Fig. 4). The proportion

of adult ticks on moderately and heavily infested moose was similar from January to April ( $G$ -test,  $P > 0.05$ ).

Newly molted adult *D. albipictus* in January were more than twice the length and width of unengorged nymphs at that time (Table I). The area of both male and female adult ticks varied between moose during each of the January, Feb-

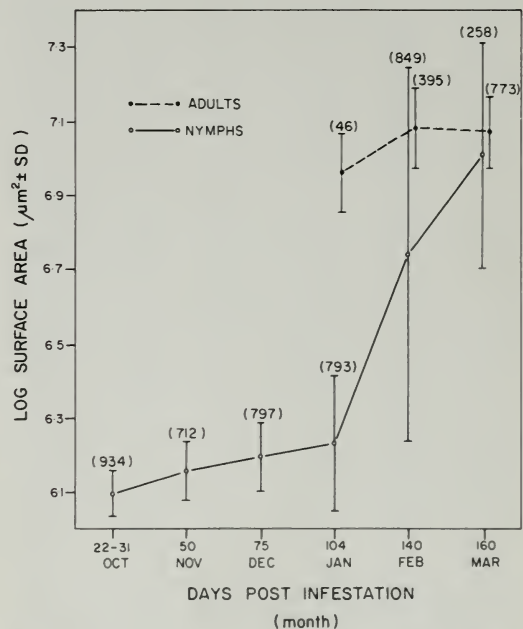


FIGURE 5. Growth of nymphs and adults of *Dermacentor albipictus* on moose. Area was calculated using the formula for the area of an ellipse.

TABLE I. *Dimensions (mm) of developing winter ticks, Dermacentor albipictus, on experimentally infested moose.*

Date	Days after infestation	Sample size		Stage of tick	Total length*	Maximum width†	Scutum length‡	Scutum width§
		Moose	Tick					
Preinfestation	0	—	193	Larva	0.60 (0.02)¶	0.49 (0.02)	—	—
22–25 October	22–25	9	36	Larva	2.14 (0.16)	1.21 (0.10)	0.20 (0.04)	0.38 (0.03)
		12	936	Nymph, unengorged	1.88 (0.11)	0.85 (0.07)	0.77 (0.04)	0.66 (0.04)
19 November	50	12	696	Nymph, unengorged	1.92 (0.11)	0.94 (0.08)	0.81 (0.06)	0.70 (0.05)
14 December	75	12	693	Nymph, unengorged	1.93 (0.10)	1.02 (0.07)	0.80 (0.05)	0.72 (0.04)
12 January	104	12	681	Nymph, unengorged	1.94 (0.01)	1.02 (0.07)	0.80 (0.05)	0.73 (0.04)
			22	Nymph, engorged	4.56 (1.11)	2.46 (0.61)	0.55 (0.17)	0.65 (0.09)
			18	Female	4.66 (0.60)	2.57 (0.34)	1.78 (0.23)	1.48 (0.14)
			28	Male	4.48 (0.58)	2.64 (0.34)	—	—
17 February	140	12	309	Nymph, unengorged	1.89 (0.12)	0.89 (0.11)	0.72 (0.05)	0.63 (0.05)
			468	Nymph, engorged	5.53 (0.80)	3.10 (0.52)	0.56 (0.08)	0.65 (0.06)
			109	Female	4.94 (0.44)	2.69 (0.28)	2.15 (0.19)	1.64 (0.15)
			287	Male	5.31 (0.63)	3.13 (0.38)	—	—
9 March	160	10	20	Nymph, unengorged	1.66 (0.30)	0.86 (0.16)	0.62 (0.08)	0.57 (0.10)
			215	Nymph, engorged	5.31 (0.61)	3.02 (0.37)	0.51 (0.08)	0.63 (0.06)
			292	Female	5.11 (0.43)	2.88 (0.26)	2.10 (0.19)	1.65 (0.12)
			4.80	Male	5.14 (0.59)	3.02 (0.39)	—	—

\* Total length = distance from the cephalic points of the scapulae to the caudal margin.  
 † Maximum width = distance across the tick at the point of greatest width.  
 ‡ Scutum length = distance from the cephalic points of the scapulae to the posterior margin of the scutum.  
 § Scutum width = distance across the scutum at the point of greatest width.  
 ¶ Mean (±1 SD).

TABLE II. *Comparison of changes in the area of unengorged nymphs of Dermacentor albipictus on moose.*

Moose*	Area (mm <sup>2</sup> )†				
	Oct	Feb	Dec	Jan	Nov
H1	1.29	1.35	1.50	1.55	1.62
H2	1.21	1.21	1.38	1.49	1.52
H3	1.24	1.28	1.49	1.60	1.63
H4	1.26	1.26	1.49	1.49	1.54
H5	1.28	1.36	1.41	1.49	1.52
H6	1.25	1.27	1.34	1.47	1.53
M1	1.28	1.35	1.44	1.61	1.65
M2	1.32	1.46	1.57	1.69	1.69
M3	1.23	1.26	1.34	1.54	1.55
M4	1.24	1.29	N/A	1.55	1.56
M5	1.20	1.35	1.46	1.55	1.55
M7	1.28	1.37	1.55	1.57	1.62

\* H = moose infested with 42,000 larvae; M = moose infested with 21,000 larvae, with 30 September as an average date of infestation.  
 † Average area calculated using the formula for an ellipse (A =  $\pi$ ILW/4).  
 Values not underscored by the same line are different (Student-Newman-Keuls,  $P < 0.05$ ).

ruary, and March collections (ANOVA,  $P < 0.05$ ). Adult males were larger than females during each of the 3 collections ( $t$ -test,  $P < 0.004$ ) (Table I). Adults grew in area from January into February (Fig. 5), but the rate of growth decreased in late February and early March (140–160 days after infestation) (Fig. 5; Table I).

Female ticks were not engorging in early to mid-March (161–164 days after infestation). Engorged females first started to detach by 24 March, and many had engorged and detached by early April (190–192 days after infestation). Engorged females that detached from moose 190–193 days after infestation weighed  $648 \pm 156$  mg.

#### Attachment of ticks

Ticks without mouthparts embedded in the hide were most common in October, February, and March, representing 3–4% of ticks sampled during that period. Almost all (99.4–99.7%) were attached during November, December, and January.

#### Effects of infestation dose on growth

The surface area of unengorged nymphs from moderately and heavily infested moose did not differ from October to February (ANOVA,  $P > 0.05$ ). The same was true of engorged nymphs in

February and March (ANOVA,  $P > 0.05$ ). Average weights of unengorged nymphs were also similar on moderately and heavily infested moose during this period. However, engorged nymphs from moderately infested moose were heavier ( $\bar{x} = 21.7$  mg) than engorged nymphs from heavily infested ( $\bar{x} = 16.8$  mg) moose ( $t$ -test,  $0.02 < P < 0.05$ ). Adult ticks of each sex were similar in area between treatment groups in both February and March. Female ticks from each treatment group were similar in weight when detached 190–192 days after infestation (ANOVA,  $P > 0.05$ ).

#### Effects of time of infestation on growth

One moose (M6) was infested 37 days later than the other 2 moose. The growth of the ticks on this moose differed slightly from that described above. Larvae grew to be as large in surface area as larvae that had infested moose earlier (Fig. 2). Unengorged nymphs from M6 were smaller than unengorged nymphs from other moderately infested moose in December and January (Scheffé's test,  $P < 0.0005$ ). However, unengorged nymphs in February and engorged nymphs in January, February, and March were similar in size, between M6 and other moose infested with moderate doses (Scheffé's test,  $P > 0.05$ ).

#### DISCUSSION

Growth and development of *D. albipictus* on moose followed a predictable pattern. Larvae grew rapidly and the parasitic larval stage was short. The nymphal stage was long, lasting until at least mid-January. Growth of nymphs was much slower than for many other ixodids. The greater proportion of nymphs loose in the hair in February and March than in November through January was coincident with nymphs molting to the adult stage. Smaller unengorged nymphs in February and March than in previous months could result if larger nymphs began to engorge earlier or if some nymphs later in the winter began to engorge when at a smaller size than was used to distinguish between engorged and unengorged nymphs. The early appearance of more adult male than female *D. albipictus* in this study has been observed in all previous studies (Bishopp and Wood, 1913; Howell, 1939b; Samuel and Barker, 1979; Osburn, 1981; Glines and Samuel, 1984).

Duration of the parasitic phase of *D. albipictus* varies between studies. Engorged female winter

ticks began detaching 22–30 days after infestation of cattle in California (Howell, 1939b) and Texas (Drummond et al., 1969). In the latter study, 70% of females detached 24–28 days after infestation (Drummond et al., 1969). Those results contrast sharply with the minimum 175 days from infestation to detachment reported in the present study. Field studies have also documented that winter ticks have a longer parasitic phase on moose in Ontario and Alberta than on black-tailed deer on Vancouver Island and on cattle in Texas (Cowan, 1946; Parish and Rude, 1946; Drummond, 1967; Addison et al., 1979; Glines and Samuel, 1984).

Adult ticks appeared more rapidly on 2 moose than on 6 other moose. This difference was likely independent of level of infestation with ticks because 1 moose was from each of the moderately and heavily infested treatment groups. Differences between individual moose may have influenced the rate of development. Differences in the rate of development of *Amblyomma maculatum* have been noted between individual hosts of the same species (Koch and Hair, 1975). Other factors could influence the rate of development of *D. albipictus*. The *nigrolineatus* form developed more rapidly than the *albipictus* form when placed on the same individual animal (Ernst and Gladney, 1975). However, the differences in rate of development between individual hosts or between the 2 forms of winter tick do not explain why the *albipictus* form on cattle in Texas required only 24 days for appearance of adults, whereas adults of the same form on moose were absent until 101–108 days after infestation (present study).

Species of hosts have also been noted to affect engorgement times of *A. maculatum* (Koch and Hair, 1975). The prolonged development of *D. albipictus* on captive moose during this study was similar to that noted on wild moose (Samuel and Barker, 1979; Glines and Samuel, 1984). However, the slower development of *D. albipictus* on moose than on cattle in Texas is not likely attributable to host-specific differences because the same general prolonged development as seen on moose was observed on cattle in Saskatchewan (Cameron and Fulton, 1926–1927).

The principal reason for a prolonged parasitic phase of winter ticks on moose probably is to synchronize the life history of the ticks with climatic patterns (see Belozarov, 1982). Although *D. albipictus* may be one of the few nearctic ixodids displaying such a morphogenetic diapause,



similar delayed development has been reported for many palearctic species of *Ixodes*, *Haemaphysalis*, and *Hyalomma* in addition to *Dermacentor marginatus* and *D. pictus* (Belozerov, 1982). Differences in rate of parasitic development of *D. albipictus* are much greater between latitudes than between hosts or longitudes, suggesting adaptations to climatic differences. The short parasitic phase of winter ticks on black-tailed deer on Vancouver Island (Cowan, 1946) may appear as an exception to this conclusion because Vancouver Island is at a comparable latitude to Ontario. However, the marine climate of Vancouver Island provides mild winters, unlike the prolonged cold periods characteristic of winters on moose range in Alberta and Ontario.

Little, if any, of the prolonged parasitic phase of *D. albipictus* on moose can be accounted for by the larval stage. The larval stage was 9–10 days long on cattle in California (Howell, 1939b) and Texas (Osburn, 1981) and 10–15 days long for the majority of larvae during this study. However, a pronounced diapause did occur during both the nymphal and adult stages on moose. The nymphal stage varied greatly in duration (41–200+ days) and was much longer than the 6–12 days observed for nymphs of *D. albipictus* on cattle in Texas (Bishopp and Wood, 1913; Osburn, 1981) and 5–13 days on horses and cattle in California (Howell, 1939a, 1939b). Similarly, the adult stage of *D. albipictus* on moose (30+ days) was much longer than the 6–8 days noted for adults on horses and 10–13 days on cattle in California (Howell, 1939a, 1939b).

Larval ticks were available for transmission to moose for 2–3 mo in Alberta (Drew and Samuel, 1985) and for a similar time in Ontario (unpubl. data). By February, larvae experimentally transmitted to moose in November were similar in size and development to larvae parasitic since early October. Larvae that attach to moose in early autumn or early winter likely both complete a normal parasitic development by having nymphal morphogenetic diapauses of varying length as occurred in this study.

Winter ticks from moose in the present study are of the *albipictus* form, which is larger than the *nigrolineatus* form of the species (Cooley, 1938; Ernst and Gladney, 1975). They were approximately 50% larger ( $\bar{x} = 0.648$  g) than replete ticks of the *nigrolineatus* form from cattle in Texas ( $\bar{x} = 0.42$  g) (Drummond et al., 1969). They were slightly larger than engorged females of the *albipictus* form from moose in Alberta ( $\bar{x} = 0.54$

g) (calculated from data in Drew and Samuel, 1986) and similar in size to replete females from mule deer in New Mexico ( $\bar{x} = 0.69$  g) (Ernst and Gladney, 1975). The smaller size of engorged female *D. albipictus* reported previously from moose in Ontario ( $\bar{x} < 0.50$  g) (Addison and Smith, 1981) is attributable to many females being partially engorged rather than replete. Geographical variation in size of other adult ixodids is well documented (Thomas, 1968; Okulova, 1981; Davey et al., 1984).

Host and geographic variation in the size of immature stages of *D. albipictus* have not been examined thoroughly. Larvae measured prior to attachment in this study are similar in size to preattachment larvae of the *nigrolineatus* form from equids and bovids in California (Howell, 1939a). However, once attached for 12 days, larval *D. albipictus* from moose were larger than replete larvae that fed on horses in California (Howell, 1939b). Amin (1969b) demonstrated that starved larvae of *D. variabilis* grew larger than larvae that were not starved. Starvation probably did not influence the size of replete larvae in the present study because larvae were available to moose on vegetation in Algonquin Park for only 2–3 wk before moose were infested. The rapid growth of larvae of *D. albipictus* from moose is characteristic of larval growth in species of *Dermacentor*, *Amblyomma*, *Boophilus*, and *Haemaphysalis* (Roberts, 1968; Amin, 1969a; Wagland et al., 1979; Barnard, 1982). The relatively short larval period of the long parasitic phase of *D. albipictus* on moose emphasizes that the functional role of larvae is transmission to a suitable host.

Engorged nymphs in the present study fell within the broad range in size of engorged nymphs of *D. albipictus* of the *nigrolineatus* form from oxen in Dallas, Texas (Bishopp and Wood, 1913). However, newly emerged nymphs of *D. albipictus* from moose are longer ( $\bar{x} = 1.89 \pm 0.16$  mm) than *nigrolineatus* nymphs of unspecified origin described by Cooley (1938) ( $\bar{x} = 1.5$  mm).

*Dermacentor albipictus* was originally described as *Ixodes albipictus* by Packard (1869), using ticks collected from moose. In the same paper Packard (1869) also described *Ixodes nigrolineatus* from deer, *Odocoileus virginianus*, in northern New York state. Banks (1908) recognized *D. albipictus* as a valid species of *Dermacentor*. Cooley (1938) synonymized *D. nigrolineatus* with *D. albipictus* following morphological study of intergrades from a variety of

areas. Hybridization of the *nigrolineatus* form from cattle in Texas and the *albipictus* form from mule deer, *Odocoileus hemionus*, in New Mexico (Ernst and Gladney, 1975) demonstrated that there was no reproductive isolation between the 2 forms and supported the synonymy of *D. nigrolineatus* with *D. albipictus*. However, biological and morphological differences between *D. albipictus* in the present and previous studies emphasize the taxonomic value of further comparative studies on hybridization and development using ticks from diverse parts of the range of *D. albipictus*.

The sizes of *Boophilus microplus* and *Amblyomma americanum* have been used as indicators of host resistance (Riek, 1962; George et al., 1985). In addition, differences in larval growth of *B. microplus* have been documented on cattle known to have varying levels of resistance to *B. microplus* (Kemp et al., 1976). The growth of *D. albipictus* was similar between moderately and heavily infested moose. This might be expected with naive (previously unexposed) moose even if moose have a demonstrable immune response to winter ticks. However, Sutherst et al. (1978) demonstrated differences in size of *B. microplus* on naive cattle given different densities of ticks. Extensive hair loss induced by grooming by moose infested with *D. albipictus* indicates that moose are responding behaviorally to tick infestations (McLaughlin and Addison, 1986). In addition, the more rapid development of adult ticks on one moose than on the other moose is suggestive of some differences in immune responses between moose. Nevertheless, from these data there is no evidence that moose may have a strong immunological response to winter ticks.

Sizes and stages of development of winter ticks can be used to determine the season of death of moose. It would be particularly easy to establish that a moose was shot or died naturally between late January and April rather than during the legal hunting season, which occurs between October and late December. Ticks are already being used as a tool in game enforcement investigations in Ontario.

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## MORPHOLOGY OF UROGENITAL SYSTEM IN FEMALE *ECHINOPARDALIS ATRATA* (ACANTHOCEPHALA)

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**ABSTRACT:** Four major areas of the urogenital system of *Echinopardalis atrata* are examined: (1) capsular protonephridial system, (2) uterine bell complex, (3) uterus, (4) vagina. Photomicrographs of cross sections from wax preparations depict morphological changes along the length of this system. Diagrammatic illustrations show relationships between excretory ducts and oviducts in the uterine bell complex. Relative positions of dorsal and ventral ligament accessory cells, median wall cells, and sheathing syncytium are also shown in this complex. The common oviduct and excretory canal join to form a ciliated urogenital canal that empties into the lumen of the uterus along the latter's anterior mid-dorsal surface. The excretory bladder is located on the inside mid-dorsal surface of the bell wall and is serviced by 2 nephridial canals—1 from each of 2 capsular protonephridia. The vagina affixes the posterior terminus of the uterus to the tegument and consists of an internal and external sphincter surrounding a hypodermal lining.

This species was originally described by Meyer (1931) as *Echinopardalis atrata* from material found in the Berlin Museum. He lists the hosts for this worm as *Herpestes ichneumon*, *Felis catus dom.*, and *Canis vulpecula*. Meyer (1931) described the female reproductive system (p. 105) in 1 sentence: "Uterusglocke und Uterus kurz, ca. 0,75 mm lang, mit kapselförmigem Protonephridialorgan am Vorderrande." Nothing is said about the male reproductive system. Meyer's figure 68 illustrated a juvenile female *E. atrata*, but it merely pointed to the area where the urogenital system was positioned without giving any details. Since its original description, this parasite has been placed in various genera. However, in 1972 Schmidt reviewed the Oligacanthorhynchidae and considered *Echinopardalis* as a junior synonym of *Oligacanthorhynchus*. Earlier, Golvan (1957), according to Schmidt (1972) had considered *Echinopardalis* a junior synonym of *Oncicola*. Proper generic placement is important because some genera in this family have dendritic, whereas others have capsular, protonephridia. Throughout these transfers between various genera, no additional descriptive information has appeared on the reproductive system of this species. The purpose of this paper is to describe the morphology of the urogenital system in female *E. atrata*.

### MATERIALS AND METHODS

*Echinopardalis atrata* were removed from dogs (*Canis familiaris*) collected in Cairo, Egypt. Infected dogs were

from the districts of Torah, El Basateen, and Maasara. Live worms from the small intestine were washed briefly in tap water before fixation in AFA or 2% glutaraldehyde. Osmotic pressure and pH were not controlled during fixation. Worms were dehydrated through a graded series of ethanols and embedded in paraplast. Sections were cut at 8  $\mu$ m and stained by standard H&E methods. This report is based on 2 sets of serial sections. The parasite orientation is based on the attachment of the dorsal ligament sac to the uterine bell. Thus, the dorsal surface of the urogenital system was clearly visible, whereas the ventral surface was covered by the ventral ligament sac.

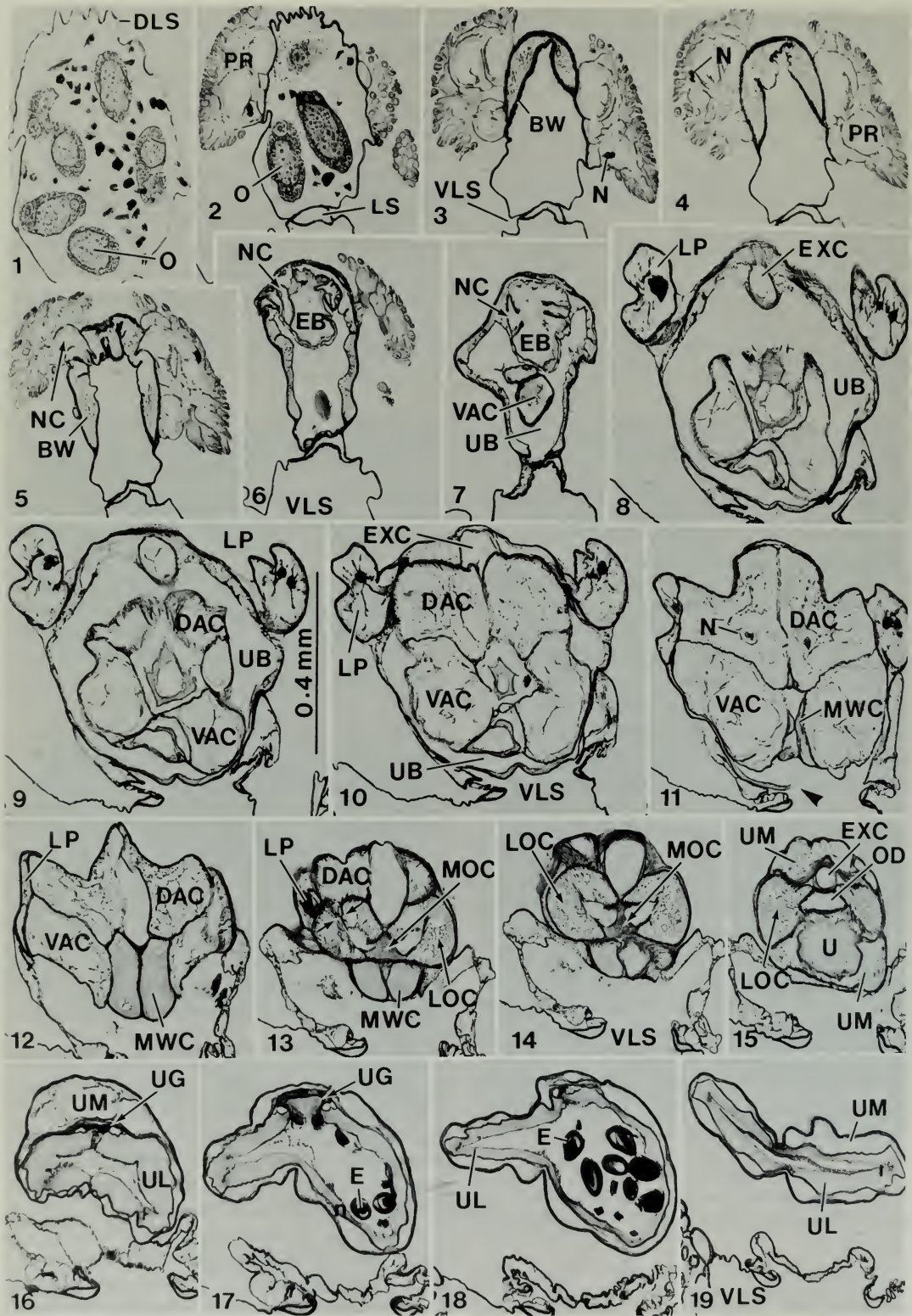
### RESULTS

Female *Echinopardalis atrata* measuring between 80 and 94 mm possess a muscular reproductive apparatus located in the posterior terminus of the body. This apparatus measures 2.5–3.0 mm in length and consists of the uterine bell complex, uterus, and vagina. A capsular protonephridium, whose products empty into the uterus by means of a urogenital canal, is also associated with this system. The complexity of this system (Figs. 1–30) prevents a ready interpretation of all events associated with egg selection and oviposition.

Mature females have dorsal ligament sacs packed with ovaries (Fig. 1) and developing eggs. Near the anterior margin of the uterine bell 2 separate groups of flame bulbs appear on the outer lateral surface of the dorsal ligament sac (Fig. 2). These flame bulbs are of a capsular nature. Each capsule has 3 nuclei. Past the uterine bell wall (Figs. 3, 4), each capsular protonephridium forms a nephridial canal (Fig. 5) that penetrates the bell wall (Fig. 6) and enters the excretory bladder (Fig. 7). The bladder lies along the inside dorsal surface of the uterine bell wall.

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The bladder is cone-shaped with the broader base anterior (Fig. 20). The bladder empties into an excretory canal located in the bell wall (Figs. 8–10). This canal may be severely compressed (Fig. 11) to the point where it is not visible, whereas a short distance away, it may be expanded to an unusually large size (Figs. 12–14). At the posterior terminus of the bell complex, the excretory canal is much smaller (Fig. 15). Note that it has maintained a dorsal position throughout and, at this level (Fig. 15), is distinct from the common oviduct. As the excretory canal and common oviduct reach the uterine lining (Fig. 16), they merge into a common duct, the urogenital canal. The latter opens into the lumen of the uterus (Fig. 17). The urogenital canal is ciliated (Fig. 17). The entire excretory canal may be ciliated. The urogenital opening into the uterus is lined by 2 separate layers. However, we have been unable to discern if both layers extend the length of the common oviduct or are restricted to the urogenital canal. The outer layer or sheathing syncytium has 2 nuclei both of which are located in tissue protruding into the uterine lumen. The terminus of this tissue may also be lobed.

The uterine bell contains 4 ligament attachment cells, although in this species the dorsal ligament sac attaches to the anterior rim of the uterine bell wall rather than to these cells. The ventral ligament attachment cells (VAC) are longer than the dorsal group and appear first in this type of preparation (Fig. 6). The organization of these 4 cells varies considerably. They may or may not form a central cavity (Figs. 9, 10) that

gradually shifts toward the ventral surface. In the latter case, a cleft along the ventral surface may shift internally anterior to the median wall cells (MWC). Each of these 4 cells has a single nucleus, 2 of which may be seen in Figure 11. These cells act as a guide for eggs ensuring their proper orientation and entry into the selector apparatus.

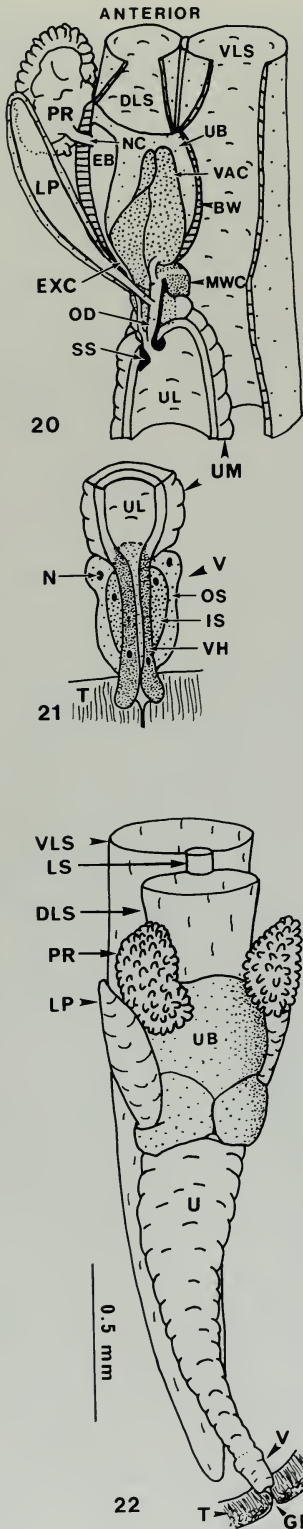
The selector apparatus (Figs. 12–15) consists of a pair of single nucleated median wall cells, a pair of single nucleated lateral oviduct cells (LOC), a single median oviduct (MOC), and a pair of single nucleated lateral bell pockets (LP). Eggs move from the anterior chamber of the uterine bell into 1 or the other of 2 lateral oviducts each formed by 1 of the LOC plus the MOC. Eggs exit this system in 1 of 3 ways: (1) into the common oviduct cell to the uterus, (2) by the MWC into the ventral ligament sac for eventual recycling, and (3) into the LP and then back to the selector apparatus where 1 of the 3 original choices will be made. Notice that the ventral wall of the uterine bell (Fig. 11, arrowhead) disappears near the anterior margins of the MWC, thus allowing free access of the eggs to the ventral ligament sac.

The LP (Figs. 8–13) are compressed against the bell wall in these sections but may appear in a variety of positions apart from the bell wall. They are attached only at the LOC through which each passes (Fig. 13, opposing arrows) to gain entry to a lateral oviduct. Their length and size depend on the number of eggs present and their state of contraction.

The uterus is about 1 mm long by 0.5 mm wide, contains 2 anteriorly located nuclei, and

FIGURES 1–19. Photographs of cross sections through anterior part of efferent duct system in female *Echinopardalis atrata*. Dorsal is the top of all figures and sequence is from anterior to posterior. 1. Dorsal ligament sac (DLS) with ovaries (O). 2. Appearance of capsular protonephridium (PR) anterior to bell wall. Ligament strand (LS) between dorsal and ventral ligament sacs (VLS). 3. Uterine bell wall (BW) first appears along dorsal surface. Nucleus (N) apparent. 4. Note that uterine bell wall forms in a dorsal to ventral direction and is very thick anteriorly. 5. Nephridial canal (NC) penetrates bell wall (BW). 6. Nephridial canal (NC) located on medial surface of bell wall and adjacent to previously formed excretory bladder (EB). 7. Nephridial canal opens into bladder; ventral ligament accessory cell (VAC) and adjacent dorsal counterpart appear in uterine bell cavity (UB). 8. Excretory canal (EC) appears in dorsal margin of bell wall. Lateral pockets (LP) appear outside, whereas dorsal ligament accessory cell (DAC) and ventral ligament accessory cell (VAC) enlarge inside UB. 9. DAC and VAC occupy much of UB. 10. DAC and VAC occupy most of UB. 11. Ventral portion of bell wall opens (arrow). Anterior portion of median wall cell (MWC) appears. EC barely visible on dorsal margin. 12. Base of uterine bell. Large MWC, much smaller DAC and VAC. 13. Two lateral oviduct cells (LDC) separated by median oviduct cell (MOC) forming 2 separate passages for eggs. Opposing arrows show point of entry for an LP. 14. VAC gone, DAC nearly gone, MWC near posterior terminus. 15. The 2 lateral oviducts have fused to form a single common oviduct (OD); outer layer of uterine muscle (UM) as well as anterior margin of inner layer (U) apparent. Note that the VLS now covers the ventral surface of UM. 16. EXC and OD fuse to form urogenital canal (UG). Uterus mostly separated from VLS. UG initiates penetration of dorsal uterine lining (UL). 17. UG empties into lumen of uterus. Cilia apparent in opening of UG. Eggs (E) present. 18. Posterior margin of opening of UG. Total separation from VLS. 19. Uterus devoid of eggs. Scale in Figure 9 applies to all figures.





consists of 2 major layers: an outer muscular layer (UM) and an inner lining (UL) (Figs. 16–19). Notice that the ventral ligament sac is reformed shortly after the appearance of the UM and remains a separate cluster to its posterior terminus. Notice also that immediately anterior to the vagina the UL differentiates (Fig. 23), forming a different type of lining for the lumen.

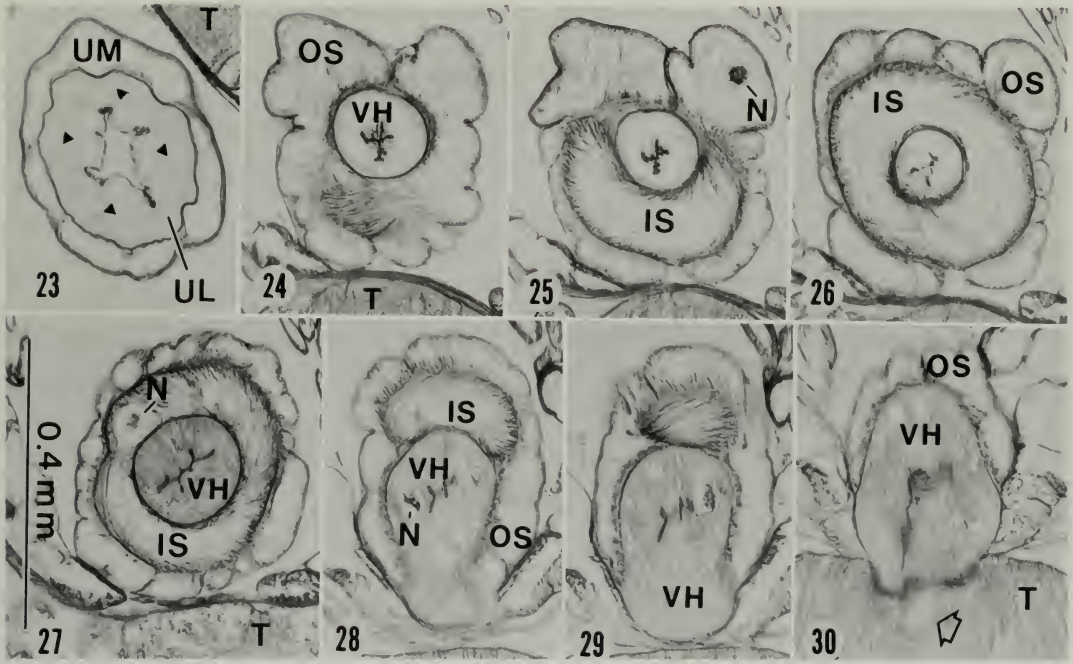
The vagina (0.4 mm long  $\times$  0.22 mm wide) (Figs. 24–30) consists of 3 major layers each with a pair of nuclei. Anteriorly, the hypodermal lining and the outer sphincter muscle appear first (Fig. 24). This is followed by the internal sphincter muscle (Fig. 25) that separates the previous layers (Figs. 26, 27) but disappears (Figs. 28, 29) prior to the association of the hypodermis with the tegument (Fig. 30). Note the beginning formation of the gonopore (Fig. 30, arrow). The vaginal hypodermis does not penetrate the tegument but is only inserted a short distance into it. The gonopore thus formed opens on the dorsal surface. A drawing of the vagina (Fig. 21) shows a lateral view of the major components.

The external appearance of the urogenital system (Fig. 22) masks the internal complexity of the egg selector apparatus. Figures 20 and 21 are diagrammatic representations showing the internal relationships of major components. Figure 22 has a dorsal flexion of the uterus. Some specimens show a ventral flexion, whereas others are terminal. These positions are apparently possible because the ventral ligament sac terminates prior to the end of the body.

## DISCUSSION

More than 1,100 species of Acanthocephala have been described, but only about a dozen au-

FIGURES 20–22. Diagrammatic sketches of efferent duct system in female *Echinopardalis atrata*. All figures organized from anterior to posterior with the ventral surface toward top of print. 20. Lateral view of uterine bell complex. 21. Lateral view of vagina. 22. Dorsal view of intact uterine bell, uterus, and vagina. BW—uterine bell wall; DLS—dorsal ligament sac; EB—excretory bladder; EXC—excretory canal; GP—gonopore; IS—internal sphincter; LP—lateral pockets; LS—ligament strand; MWC—median wall cell; N—nucleus; NC—nephridial canal; OD—common oviduct; OS—outer sphincter; PR—capsular protonephridium; SS—sheathing syncytium; T—tegument; U—uterus; UB—uterine bell; UL—uterine lining; UM—outer uterine muscle; V—vagina; VAC—ventral ligament attachment cell; VH—vaginal hypodermis; VLS—ventral ligament sac.



FIGURES 23–30. Photographs of cross sections through posterior terminus of reproductive system of *Echinopardalis atrata* emphasizing vaginal organization. 23. Uterus immediately anterior to vagina. Notice inner lining of uterus (UL) seems to have a distinct layer (arrowheads) adjacent to lumen. 24. Outer vaginal musculature showing outer sphincter (OS) surrounding vaginal hypodermis (VH) adjacent to a dorsal portion of tegument (T). 25. Appearance of inner sphincter muscle between OS and VH. Nucleus (N) on ventral surface of OS. 26. Inner sphincter completely surrounds VH separating it from OS. 27. Section immediately prior to initial loss of IS. 28. IS no longer encircles VH, which has interrupted OS along dorsal surface. 29. VH contacts tegument. 30. VH penetrates a portion of tegument (T) that begins to separate (arrow). Scale in Figure 27 applies to all figures.

thors have published detailed studies on the female efferent duct system. However, many have illustrated this system from whole-mount preparations. Most of the latter add little to our understanding of this complex, but some, such as Machado Filho's (1950) description of *Prosthenorchis freitasi*, are very helpful. Kaiser (1913) presented a good review of the earlier literature including his 1893 work in which he described the histology of 6 different efferent duct systems. More recent studies (Whitfield, 1968; Asaolu, 1980) have reviewed the literature since Kaiser, along with their descriptions of the histology of the female reproductive system in *Polymorphus minutus* and *Moniliformis moniliformis*, respectively.

Most of our current information is based on species belonging to the Archiacanthocephala or Palaeacanthocephala. Little is known about efferent ducts in female Eoacanthocephala. Among Archiacanthocephala, *Echinopardalis atrata* closely resembles other members of the Oliga-

canthorhynchidae, all of which have a protonephridial excretory system associated with the efferent duct system. In this family good descriptions are available for *Macracanthorhynchus hirudinaceus* by Kaiser (1893), *Oligacanthorhynchus thumbi* by von Haffner (1942), and *Oligacanthorhynchus microcephala* by Kilian (1932). However, only *E. thumbi* has a capsular type protonephridium like *E. atrata*. Von Haffner (1942) illustrated the uterine bell complex by 4 line drawings of cross sections. These drawings (pp. 222–273) have many of the characters observed in our Figures 10, 14, and 15. However, von Haffner labeled only a few components and, therefore, limited the view of this system. Von Haffner did indicate that the excretory canal was ciliated throughout its length. Moreover, he did not accept the view that an excretory bladder as such existed but believed that this was simply a nonciliated anterior portion of the excretory canal that was capable of expansion.

Descriptions of bell complexes by Kaiser



(1893) for *M. hirudinaceus* and Kilian (1932) for *O. microcephala* differ from *E. atrata* by the presence of "Plasmastreifen" and much smaller ligament attachment cells. The organization of the excretory ducts is similar in all 3, but *E. atrata* has a capsular rather than a dendritic protonephridium.

Asaolu's (1980) report on the reproductive system of *M. moniliformis* (Archiacanthocephala: Moniliformidae) added considerable information to earlier work (Yamaguti and Miyata, 1942) on this species. Major components of this system resemble those described here, but clear differences exist beyond the absence of an excretory system in *M. moniliformis*. For example, Asaolu described only 2 cells extending into the bell chamber, whereas there are 4 such cells in *E. atrata*. He also described a nucleated ligament attachment syncytium extending from anterior of the uterine bell to the base of the bell chamber, which is absent in *E. atrata*. Other differences occur in the size and shape of the uterus and vagina.

Terminology used to describe the components comprising the efferent duct varies widely; see von Haffner (1942) and Whitfield (1968). There seems little consensus on terms used for cells along the route for egg passage. For example, we use oviduct cells, whereas Whitfield (1968) used lappets; we use median oviduct cells, whereas he used sheathing syncytium. We use sheathing syncytium for the outer cellular lining of the common oviduct that empties into the uterus. Our interpretation of this material suggests that the tissue (MOC; Figs. 13, 14) medial to the 2 lateral oviducts is separate from that forming the outer covering (SS) of the common oviduct. The outer covering may extend into the lumen of the uterus in *E. atrata* where it becomes lobed. The overall appearance of this extension resembles that described for *P. minutus* by Whitfield (1968), except that in *E. atrata* 2 nuclei are located in that portion of the syncytium extending into the lumen.

The uterus in all species seems to be constructed on the same plan differing only in size and shape. However, the differentiation of the posterior most inner lining into 2 parts (Fig. 23) suggests the appearance of a function different from that tissue anterior to this point. The appearance of this area is compatible with a secretory function, but this view is based only on morphology. The vagina in different species has

not been as uniform as the uterus. The 3 layers of tissue comprising the vagina in *E. atrata* seem to occur in most Acanthocephala. There are no vaginal glands as described for *Acanthogyrus antispinus* (Verma and Datta, 1929) or additional sphincters as described for *M. moniliformis* by Asaolu (1980). Nevertheless, the number and general location of nuclei agree with the report of Whitfield (1968) for *P. minutus*.

#### ACKNOWLEDGMENTS

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## SYNLOPHE OF *NEMATODIRUS NEOTOMA* (TRICHOSTRONGYLOIDEA)

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**ABSTRACT:** The synlophe of *Nematodirus neotoma* from *Neotoma* spp. is characterized. The cervical synlophe is composed of 30-32 and 36-42 ridges in males and females, respectively. Of these, 14 and 20-22 ridges are continuous in the cervical zone and extend to the base of the cephalic expansion. Six pairs of lateral ridges are discontinuous but extend greater than one-third the length of the cervical region. In both males and females, the number of ridges increases posteriad, terminating near the bursa in the male, and extending the entire length of the body in the female. The synonymy of *N. neotoma* and *N. tortuosus* was confirmed.

Studies have indicated the importance of the synlophe as a character in trichostrongyloid systematics (Durette-Desset, 1971, 1985; Lichtenfels and Pilitt, 1983; Rossi, 1983; and others). Among *Nematodirus* spp. (Trichostrongyloidea: Nematodirinae), detailed studies of the synlophe have largely been limited to those species parasitic in ruminants (Durette-Desset, 1978, 1979; Lichtenfels and Pilitt, 1983; Rossi, 1983; Hoberg et al., 1986; Hoberg and Rickard, 1988).

*Nematodirus neotoma* Hall, 1916, and *N. tortuosus* Tucker, 1941, were originally described from woodrats in Colorado (*Neotoma desertorum* Cary, *N. mexicana fallax* Merriam, *N. floridana baileyi* Merriam, and *N. cinerea rupicola* Allen) and California (*N. lepida intermedia* Rhoads and *N. fuscipes macrotis* Thomas), respectively (Hall, 1916; Tucker, 1942). Miller and Schmidt (1982) reduced *N. tortuosus* as a synonym of *N. neotoma*.

Although *N. neotoma* can be differentiated from its congeners based on current descriptions, the synlophe and cephalic structures were never defined in previous studies. In the study reported herein, the synlophe and cephalic structures of *N. neotoma* are described, and the synlophe is compared with that of other *Nematodirus* spp. The occurrence of *N. neotoma* in a host-group not typical of its congeners is discussed with references to parasite morphology and parasite-host

biogeography. Additionally, the synonymy of *N. neotoma* and *N. tortuosus* is confirmed based on characteristics of the synlophe in the cervical region.

### MATERIALS AND METHODS

The description of the synlophe and cephalic structures is consistent with the methodology and terminology presented by Lichtenfels and Pilitt (1983). In the description, the cervical region is the zone extending anteriad from the cervical papillae and excretory pore to the posterior margin of the cephalic expansion. Specimens of *Nematodirus neotoma* were examined using interference contrast microscopy (Leitz) and scanning electron microscopy. Prior to examination, material for light microscopy was transferred to 70% ethanol/5% glycerine and cleared in glycerine by evaporation. Ten specimens of each sex were prepared as temporary whole mounts, and 5 specimens of each sex were studied in transverse sections cut by hand with a scalpel blade. Eight male and 7 female specimens were examined using SEM (stubs submitted to the USNM Helm. Coll. No. 75507). All measurements are in  $\mu\text{m}$  unless stated otherwise.

*Specimens examined:* *Nematodirus tortuosus*: 2 male specimens (type) from *Neotoma* spp. at West Los Angeles, California, USNM Helm. Coll. No. 36717. *Nematodirus neotoma*: male and female specimens from *Neotoma cinerea rupicola* at northern Weld County, Colorado, and southeastern Laramie County, Wyoming, 15 October 1979 (borrowed from collection of G. D. Schmidt, Nos. GK-7 and GK-8).

### RESULTS

#### Cuticular morphology of *N. neotoma*

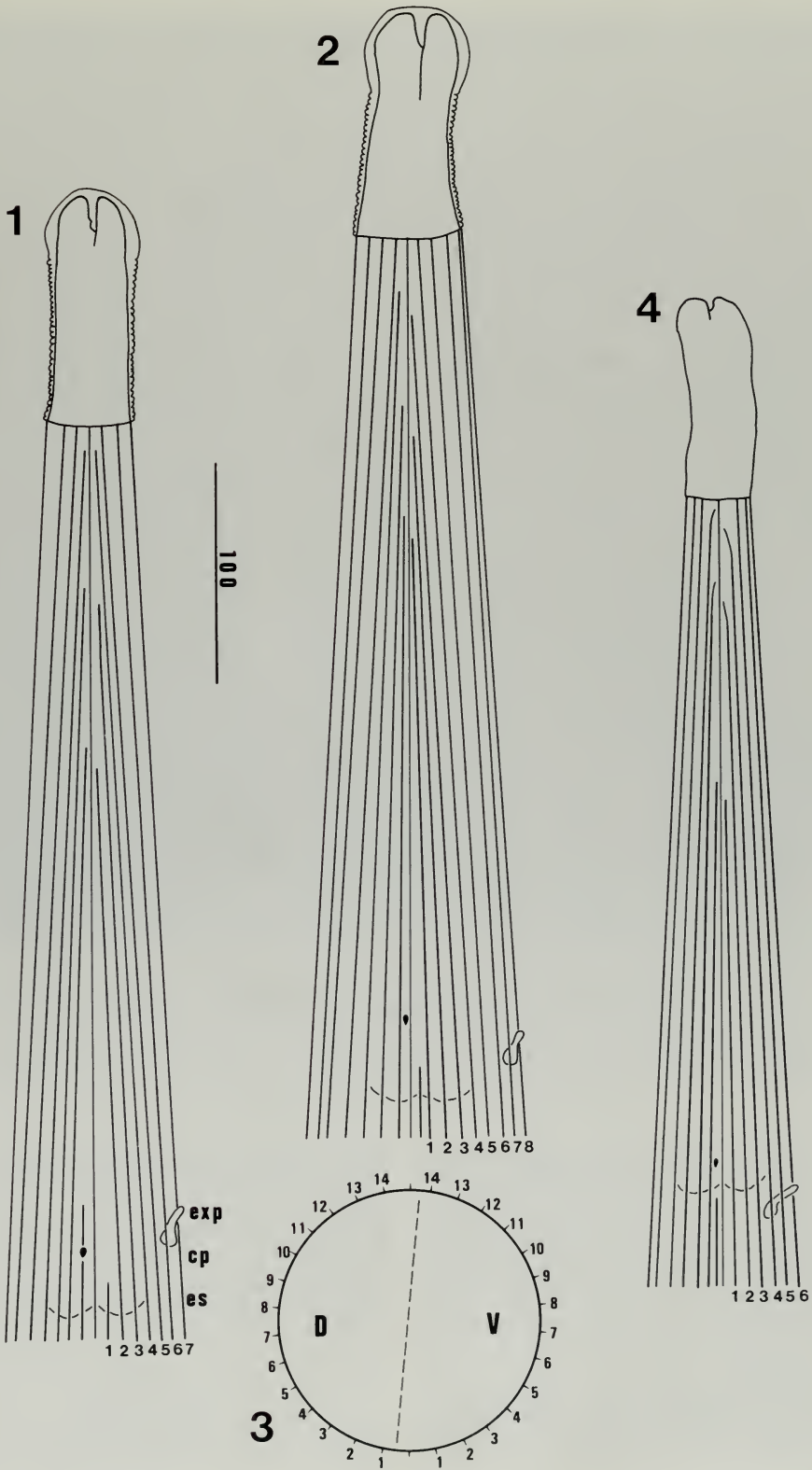
(Figs. 1-16)

*Synlophe:* At the level of the esophagus and cervical papillae, the synlophe of *N. neotoma* consists of a bilaterally symmetrical system of 30-32 ridges in males and 36-42 ridges in females. The fine lateralmost cervical ridges originate 1,000-1,500 from the cephalic

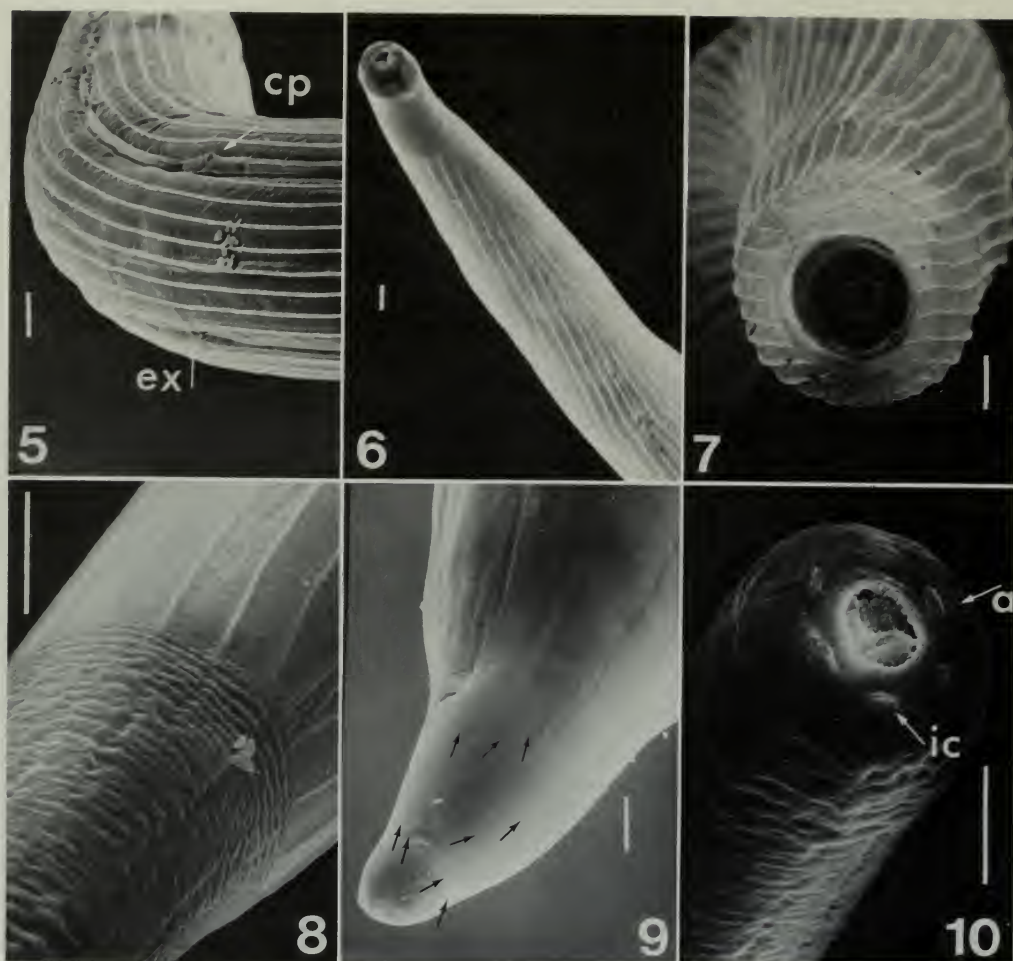
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FIGURES 1-4. Synlophe of *Nematodirus neotoma*. Scale = 100  $\mu\text{m}$ , same for Figures 1, 2, and 4. 1. Male, cervical synlophe, right lateral view (excretory pore = exp, cervical papilla = cp, and esophagus = es). Origin of fine lateralmost ridge not shown. 2. Female, cervical synlophe, right lateral view. 3. Schematic of transverse section showing synlophe at level of cervical papilla and esophagus in male (not to scale). 4. Synlophe of *N. tortuosus* (= *N. neotoma*), right lateral view in male (type).







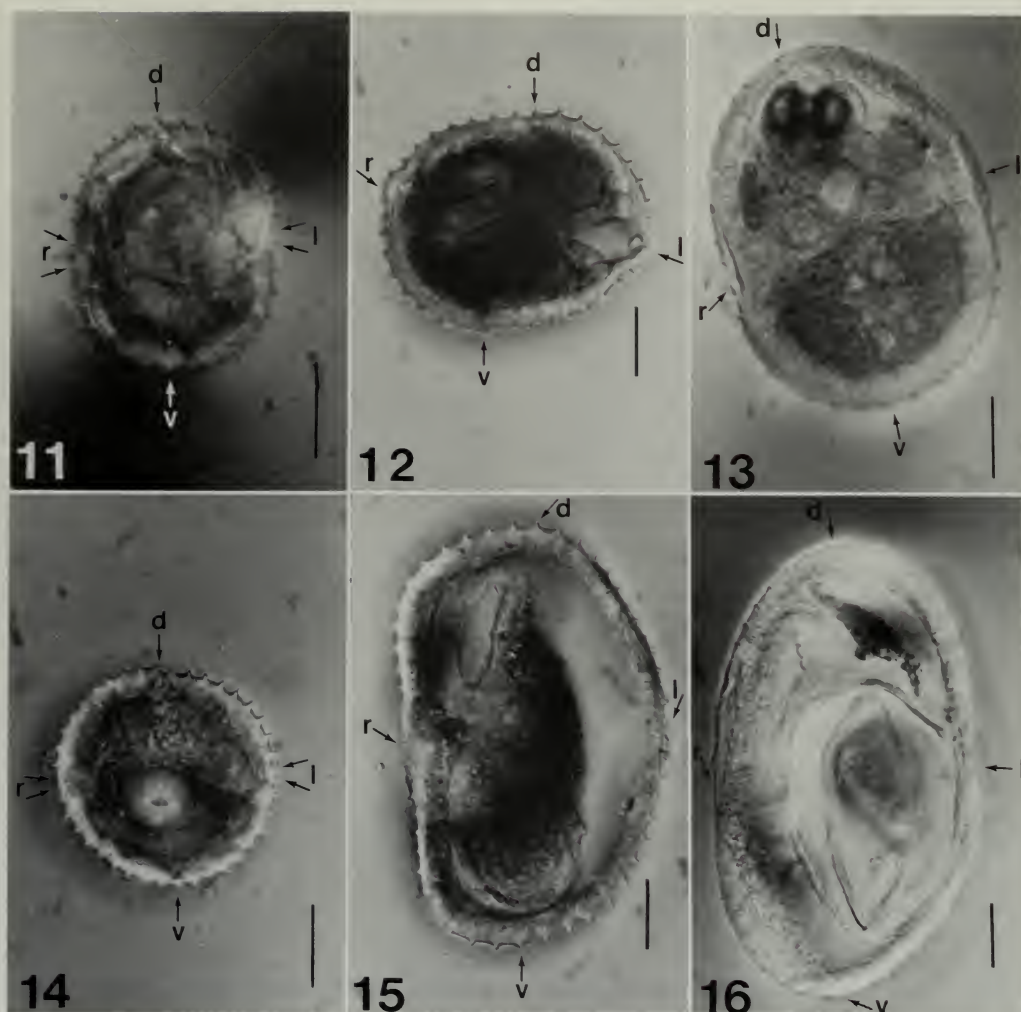
FIGURES 5–10. Scanning electron microscopy of *N. neotoma*. All scales = 10  $\mu$ m. 5. Cervical synlophe, left lateral view, showing left cervical papilla (cp) and excretory pore (ex). 6. Cervical synlophe, right lateral view, showing pattern of discontinuous ridges anterior to cervical papilla. 7. En face view of female, showing 20 continuous ridges extending to the base of the cephalic expansion. 8. Detail of synlophe at base of cephalic expansion showing termination of cervical ridges and transverse striations on cephalic expansion. 9. Caudal extremity of female showing minute cuticular ridges adjacent to tail extending posteriad to the vulva. Arrows indicate points of termination for individual ridges. 10. En face view of cephalic extremity of female showing perioral denticles, papillae of inner circle (ic), and amphids (a).

expansion. The right lateral ridge is occasionally interrupted at the level of the cervical papilla. The seventh and eighth ventral ridge is interrupted at the excretory pore in males and females, respectively. Fourteen ridges in males and 20–22 ridges in females are continuous in the cervical zone and terminate at the base of the cephalic expansion. Six pairs of lateral ridges extend greater than one-third the cervical distance but terminate before reaching the cephalic expansion. All ridges are perpendicular and there is no gradient in height.

Posteriad from the cervical zone the numbers of ridges increase in both males and females. Slightly anterior to the midbody in males there are 35–36 ridges; at the midbody, 39–40. At the level of the proximal end of

the spicules, there are 47–49 ridges; dorsally, the ridges become markedly reduced in height. The synlophe terminates 350–400 from the bursa. At the midbody in females, the synlophe is composed of 47–55 ridges; maximum observed posterior to this region is about 60 ridges. Within a distance of 500 anterior to the vulva, the dorsal ridges become reduced in height. Although markedly attenuated posterior to the vulva, and not generally discernible with light microscopy, the synlophe extends to the caudal extremity of females.

**Cephalic structures:** Cephalic expansion longer than wide, with slight swelling in anterior and distinct transverse striations in posterior. Papillae of internal circle 6 in number, with sclerotized semicircular supports. Papillae of the external circle indistinct. Perioral den-



FIGURES 11–16. Transverse sections of *N. neotoma* showing distribution of cuticular ridges. All figures are oriented with dorsal toward top; arrows indicate dorsal (d), ventral (v), and lateral ridges (l = left; r = right); all scales = 30  $\mu$ m. 11. Cervical region of male with 32 ridges; note small ridges laterally. 12. Midbody region of male with 39 ridges. 13. Posterior region of male at level of proximal end of spicules showing 43 ridges. 14. Cervical region of female with 41 ridges. 15. Midbody region of female (cut at 11.5 mm from anterior in specimen measuring 23.18 mm) showing 53 ridges. 16. Posterior to midbody (12.4 mm from anterior) showing 54 ridges; synlophe is no longer easily discernible in interference contrast beyond this point.

ticles 42–52 (46) ( $n = 4$ ) in number for males, 46–59 (52) ( $n = 7$ ) for females.

**Comparison with *Nematodirus tortuosus*:** The numbers of ridges in the type specimens of *N. tortuosus* could not be accurately determined. However, the pattern of the cervical synlophe, particularly the extent of the lateral ridges and origin and form of the lateralmost ridge appeared identical to that observed in specimens of *N. neotoma*.

#### DISCUSSION

The number and longitudinal extent of ridges comprising the synlophe of *N. neotoma* in the

present study, as determined by light microscopy, was generally similar to that reported previously. Although the number of perioral denticles was greater (42–52 for males; 46–59 for females) than that reported by Durette-Desset (1979), this and other differences in the numbers of ridges are considered as intraspecific variation. Based on these characters, the synonymy of *N. neotoma* and *N. tortuosus* is confirmed.

Previous descriptions of *Nematodirus* spp. from cricetids did not consider details of the synlophe. Hall (1916), in describing *N. neotoma*, reported



the presence of longitudinal lines in males and females. Tucker (1942) described longitudinal ridges throughout the length of males of *N. tortuosus*, whereas in females, the synlophe was confined to the region anterior to the vulva. Durette-Desset (1979) elucidated some details of the synlophe in *N. tortuosus*. The numbers of ridges increased posteriad attaining a maximum of 40–56 in males and 52–61 in females. The synlophe terminated within 400  $\mu$ m of the vulva in females and 1,000  $\mu$ m from the bursa in males. Additionally, about 40 perioral denticles were enumerated. Recently, Miller and Schmidt (1982) reported 22–48 and 26–68 ridges in males and females of *N. neotoma*, respectively (presumably a range from anterior to posterior). The synlophe in female specimens was observed to extend posteriad to the vulva.

Considering the other species of *Nematodirus* previously examined, *N. neotoma* is characterized by a substantially greater number of cervical and midbody ridges. The synlophe in the cervical region (anterior to the cervical papillae) has been characterized for only 8 species of *Nematodirus* that are parasites of the Bovidae (Lichtenfels and Pilitt, 1983; Hoberg et al., 1986; Hoberg and Rickard, 1988). A bilateral system of 16 ridges was found in *N. maculosus* Becklund, 1965, an 18-ridge system was typical of *N. filicollis* (Rudolphi, 1802), *N. davtianii* Grigorian, 1949, *N. oratians* (Raevskaia, 1929), *N. abnormalis* May, 1920, *N. spathiger* (Railliet, 1896), and *N. battus* Crofton and Thomas, 1951, and 26 ridges were reported for *N. helvetianus* May, 1920 (lateral-most extending less than one-third of the cervical distance). The midbody region has been examined in 16 species of *Nematodirus* from the Bovidae, Cervidae, and Camelidae (Becklund, 1963; Durette-Desset, 1978, 1979; Rossi, 1983). Among these only 4 species have 30 or more ridges at the level of the midbody, viz. *N. helvetianus* (30–36) from bovids, *N. europeus* Jansen, 1972 (33–36), *N. roscidus* Railliet, 1911 (34), from palearctic cervids, and *N. lamae* Becklund, 1963, from neotropical camelids (approximately 40). Additionally, *N. odocoilei* Becklund and Walker, 1967, from nearctic cervids has 34–42 ridges at the midbody (Hoberg, unpubl. data).

Species of *Nematodirus* generally are parasites of the Artiodactyla and only 2 species are known from rodents (see Kulmamatov, 1974; Durette-Desset, 1979). In addition to *N. neotoma* from *Neotoma* spp., *N. mugosaricus* Schulz, 1926, was

described from *Citellus* in the Soviet Union (Skrjabin et al., 1954). The distribution of these species appears to represent independent events of colonization of cricetids and sciurids by species of *Nematodirus* typical of ruminants (see Durette-Desset, 1979).

The widespread distribution of *N. neotoma* among *Neotoma* spp. in western North America could indicate that *Nematodirus* became parasites of woodrats prior to geographic isolation resulting in speciation and subspeciation of the host-group in the Pleistocene (see Kurtén and Anderson, 1980). Morphologically, the synlophe of *N. neotoma* appears most similar to some species characterized by a high number of ridges from camelids or cervids (Rossi, 1983; Hoberg, unpubl.). Current evidence thus suggests a possible switch from either of these host-groups to *Neotoma*. Additional study involving phylogenetic analyses will be required to evaluate the degree of relationship among *N. neotoma* and other *Nematodirus* spp.

#### ACKNOWLEDGMENTS

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## A BUCEPHALID LARVA, *CERCARIA PLEUROMERAE* N. SP. (TREMATODA: DIGenea), PARASITIZING A DEEPWATER BIVALVE FROM THE GULF OF MEXICO

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**ABSTRACT:** A new bucephalid larva, *Cercaria pleuromerae*, is described from bivalve molluscs, *Pleuromeris armilla*, taken from the Gulf of Mexico, 185 mi southeast of New Orleans, Louisiana, U.S.A., at a depth of 205 m. Sporocysts occurred in the gonads and digestive gland, apparently preventing gametogenesis in the host. Cercariae emerged freely and continually in both day and night and attached to one another by means of their caudal furcae to form a multicercarial net. This net may serve to increase the probability of contact with the second intermediate host, which is probably a demersal forage fish.

Of the more than 30 marine bucephalid larvae known to date, only 5 have been reported from the Gulf of Mexico (Table I), all parasitizing bivalved molluscan hosts as is characteristic of all known cercariae of the family.

The bucephalid cercariae previously reported have been collected from hosts inhabiting relatively shallow water. The present report describes a new bucephalid larva from a bivalve host inhabiting the deeper waters of the Gulf of Mexico, and represents the greatest depth from which clams parasitized by bucephalid larvae have yet been reported.

### MATERIALS AND METHODS

Three specimens of the host clam, *Pleuromeris armilla*, were collected in the northeastern Gulf of Mexico approximately 185 mi southeast of New Orleans, Louisiana, U.S.A. (28°14'N, 88°7.6'W) at a depth of 205 m, using a 20' otter trawl of 1" mesh net with a bag liner of ¼" mesh. The clams were kept in iced seawater for 24 h prior to examination.

Cercariae emerging from clams isolated for 48 hr in 30‰ seawater at room temperature were observed alive in seawater and stained alive with vital dyes. Cercariae were heat-killed in seawater prior to measurement. Infected clams were subsequently opened to obtain developmental stages, which were examined in seawater, then fixed in hot formalin-acetic acid-alcohol, and stored in 70% alcohol for measurement and reference.

Unless otherwise specified, measurements are expressed in  $\mu$ m and ranges are followed by means in parentheses.

### RESULTS

Of the 3 specimens of *Pleuromeris armilla* obtained, 2 that measured 10 and 10.5 mm in diameter, were found to be infected with buceph-

alid larvae. The third specimen, 9 mm in diameter, was an uninfected ripe female. The bucephalid larva is named for the genus of the host clam and is described below.

### DESCRIPTION

#### *Cercaria pleuromerae* n. sp.

*Host:* *Pleuromeris armilla* (Dall, 1902) (Bivalvia, Carditidae).

*Infected tissues:* Gonad and digestive gland.

*Incidence of infection:* Two of 3 specimens.

*Locality:* Northeastern Gulf of Mexico, 29°14'N, 88°7.6'W, at 205 m depth.

*Type specimen:* #80292 deposited in the Helminthological Collection of the U.S. National Museum, Beltsville, Maryland.

#### Cercaria

Body of naturally emerged cercariae (Fig. 1) 143-175 (153) long, 35-50 (42) wide, roughly oval in cross section, containing numerous oval cystogenous glands evenly distributed in parenchyma. Tegument uniformly minute spinose.

Tail stem 28-31 (30) long, 48-58 (53) wide; weakly indented posteriorly to form 4 lobes. Caudal furcae arising from anterolateral faces of tail stem, extending from 5 to over 20 times length of body.

Anterior penetration organ 20-25 (23) long and 17-20 (19) wide, containing about 5 pairs of penetration glands with ducts opening anteriorly at a common sub-terminal pore surrounded by 4 anterior liplike structures.

Pharynx 12-16 (14) in diameter, opening on ventral surface of posterior half of body; distance from its anterior margin to the anterior end of the body being 60-70% of total body length. Intestine extending dorsally and anteriorly and bending posteriorly somewhat below level of pharynx, its contents often staining red with neutral red stain.

Genital anlagen an indistinct mass of neutral red-staining tissue between intestine and posterior end of body.

Excretory bladder I-shaped, opening posteriorly through duct entering the upper part of the tail stem;

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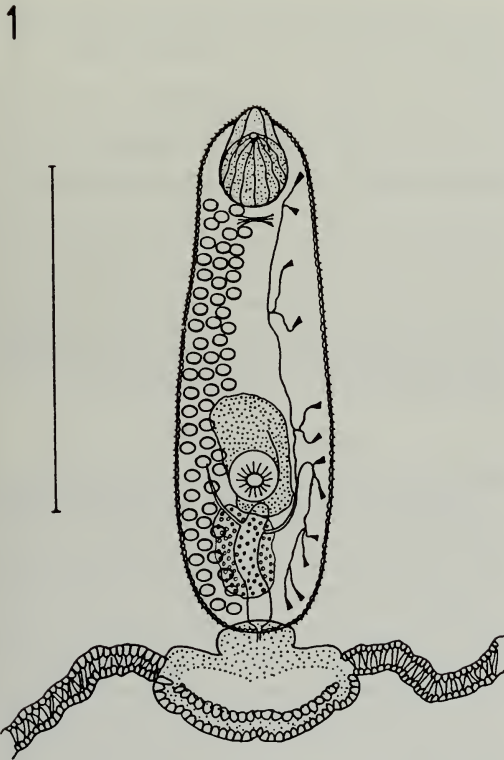


FIGURE 1. *Cercaria pleuromerae*, ventral view. Scale bar = 100  $\mu$ m.

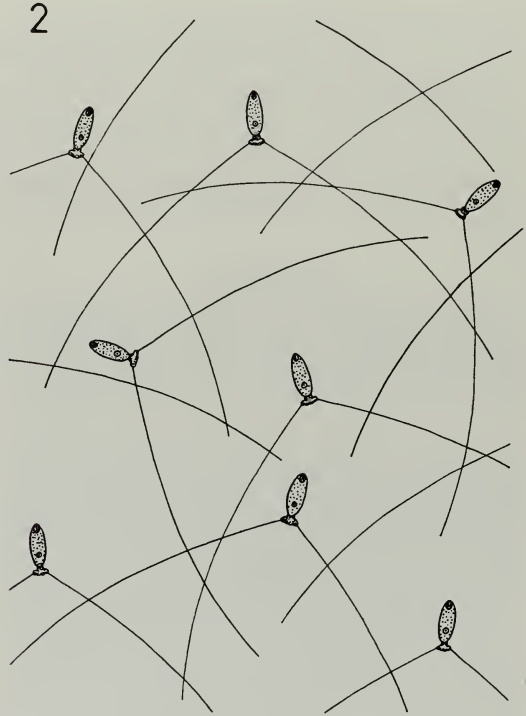


FIGURE 2. *Cercaria pleuromerae*, portion of multicercarial net.

joined laterally and subterminally by main excretory ducts dividing at pharyngeal level.

Flame cell formula  $2[(2 + 2 + 2) + (2 + 2 + 2)] = 24$ .

Cercariae emerged continually, both day and night, from infected clams isolated in 30‰ seawater at room temperature. Some individual cercariae were found resting singly on the bottom of the container constantly extending, coiling, and retracting their long caudal furcae. Most, however, were observed with their furcae extended and fixed in a gently convex curve and firmly attached to the furcae of other cercariae at fairly regular intervals as shown in Figure 2. This, in effect, formed a net of cercariae that was neutrally buoyant and that extended up from the bottom of the container into the water column. The net remained intact when agitated with a dissecting needle. The bodies of cercariae that were thus enmeshed appeared to be in the contracted

state and were not observed to elongate and contract alternately as did isolated cercariae. There also appeared to be some degree of polarization to the net in that the bodies of enmeshed cercariae were generally oriented toward the same downward direction.

### Sporocyst

The gross aspect of the gonad and digestive gland areas of infected clams had an overall light cream coloration and appeared to be somewhat dry and coarse in texture as compared to the smooth and glistening-wet appearance of this area in the single uninfected specimen of *Pleuromeris armilla*, which was available for comparison. The gonadal area of infected clams was devoid of gametes and sex could not be determined, indicating that the infection prevented gamete formation. The single uninfected host clam examined was a ripe female that was smaller than both the infected specimens.

TABLE I. *Bucephalid larvae parasitizing bivalves from the Gulf of Mexico.*

Host bivalve	Locality	Name assigned	Reference
<i>Crassostrea virginica</i>	Bay St. Elaine, Louisiana	<i>Bucephalus cuculus</i> McCrady, 1873	Menzel and Hopkins, 1955
<i>Crassostrea virginica</i>	Lake Calcasieu, Louisiana	<i>Bucephalus cuculus</i> McCrady, 1873	Turner, 1985
<i>Donax variabilis</i>	Port Aransas, Texas	<i>Bucephalus loeschi</i> Hopkins, 1958	Hopkins, 1958
<i>Ostrea equestris</i>	Port Aransas, Texas	No name assigned	Hopkins, 1956
<i>Pinna carnea</i>	Tortugas, Florida	<i>Cercaria</i> "N" Miller, 1925	Miller, 1925
<i>Mulinia lateralis</i>	Apalachicola Bay, Florida	<i>Cercaria apalachiensis</i> Holliman, 1961	Holliman, 1961



Sporocysts were light cream-colored and uneven in diameter, having irregularly alternate swellings and constrictions, being 5–16 in diameter in heat-killed specimens. Sporocysts were branched in typical bucephalid fashion and up to 300 in total length. Tips of the sporocysts tapered to a point and were capped with a zone of undifferentiated cells.

Germ balls and numerous cercariae in various stages of development were visible through the sporocyst tegument.

### DISCUSSION

*Cercaria pleuromerae* bears close morphological resemblance to 3 species of marine bucephalid cercariae that have flame cell formulae of  $2[(2 + 2 + 2) + (2 + 2 + 2)] = 24$ , similar excretory bladders, and similar anterior organs. The cercaria of *Bucephalus cuculus* McCrady, 1874, as redescribed by Hopkins (1954), differs from *C. pleuromerae* in being generally larger in size (240–400  $\mu\text{m}$ ) and in having a tail stem that is not distinctly divided into posterior lobes.

The cercarial stage of *Parabucephalopsis prosthorchis* Tang and Tang, 1976, is also similar to *C. pleuromerae*, however, it is larger (400  $\mu\text{m}$ ), has a more centrally placed pharynx, and has an excretory duct that enters the tail stem where it bifurcates and terminates anterolaterally via 2 separate pores.

*Cercaria caribbea* XLII Cable, 1956 (redescribed by Cable, 1963), appears to be most similar to *C. pleuromerae*. It differs in the more anterior position of the gut relative to the pharynx and in the possession of 6 setae on the posterior margin of the tail stem that are lacking in *C. pleuromerae*. The body of *C. caribbea* XLII is also slightly larger than *C. pleuromerae*, being 190–200  $\mu\text{m}$  in length.

The cercarial net formation seen in *C. pleuromerae* may serve to increase the chances of cercariae contacting and successfully penetrating the second intermediate host, which is probably a deepwater demersal forage fish. This phenomenon may also occur in the cercariae of other bucephalid species and may account for the even spatial distribution often encountered in bucephalid metacercarial infections.

A previous report of cercarial association is that of the "Rattenkonig" (rat king) type of cercaria (Ward and Whipple, 1918), but this type differs from the present species in not being gas-

terostomatous (bucephalid) and in that the single furcae are all united at their tips at a common point.

### ACKNOWLEDGMENTS

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## NEOTROPICAL MONOGENEA.

### 13. *RHINONASTES PSEUDOCAPSALOIDEUM* N. GEN., N. SP. (DACTYLOGYRIDAE, ANCYROCEPHALINAE), A NASAL PARASITE OF CURIMATÁ, *PROCHILODUS NIGRICANS* AGASSIZ (CYPRINIFORMES, PROCHILODONTIDAE), IN BRAZIL

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**ABSTRACT:** *Rhinonastes pseudocapsaloideum* n. sp. (Dactylogyridae, Ancyrocephalinae) is described from the nasal cavity of *Prochilodus nigricans* Agassiz (Cypriniformes, Prochilodontidae) in Brazil. *Rhinonastes* n. gen. is proposed for species possessing a dextroventral genital pore, a bilobed testis, a ventral C-shaped ovary lying between the 2 testicular lobes, and a disc-shaped haptor armed with a ventral anchor-bar complex and 14 hooks.

The nasal cavity as a site of infestation for Monogenea has been poorly investigated. Only 3 species of Dactylogyridae have been described from the nasal cavity of Neotropical freshwater fishes: *Rhinoxenus piranhus*, *R. arietinus*, and *R. nyttus*, all by Kritsky et al. (1988), occur in characid fishes inhabiting the Brazilian Amazon. During an investigation of copepod parasites from the nasal cavity of Amazonian fishes by V.E.T., 8 specimens of an undescribed species of Dactylogyridae were collected from the nasal cavity of the characid *Prochilodus nigricans* Agassiz (Prochilodontidae) from Brazil. This form is described herein as the only member of the new genus *Rhinonastes*.

#### MATERIALS AND METHODS

Specimens were collected using the methods described by Kritsky et al. (1988). Preparation of helminths for study followed the procedures outlined by Kritsky et al. (1986). In addition, some specimens were stained with eosin and orange G in 95% ethyl alcohol and mounted in Harleco synthetic resin (available from American Scientific Products) to determine internal anatomy. Measurements, all in  $\mu\text{m}$ , were made with the aid of a filar micrometer, except length of the cirrus was approximated using a Minerva curvimeter on camera lucida drawings. Yamaguti's (1963) classification of the Dactylogyroidea is used as basis for assignment of helminths. Type specimens were depos-

ited in the helminth collections of the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Amazonas, Brazil; the U.S. National Museum (USNM), USDA, ARS, Agricultural Research Center-East, Beltsville Maryland; and the University of Nebraska State Museum (HWML), University of Nebraska, Lincoln.

#### DESCRIPTION OF TAXA

##### *Rhinonastes* n. gen.

**Diagnosis:** Dactylogyridae, Ancyrocephalinae. Body robust, divisible into cephalic region, trunk, peduncle, haptor. Tegument thin, smooth. Head organs, cephalic lobes present; cephalic glands unicellular, comprising 2 bilateral groups posterolateral to pharynx. Eyes present. Mouth subterminal, midventral; pharynx muscular, glandular; esophagus present; intestinal ceca (2) confluent posterior to gonads, lacking diverticula. Gonads overlapping, intercecal; testis dorsal to ovary, bilobed; ovary C-shaped, with ends directed dorsally. Vas deferens looping left cecum; seminal vesicle a dilation of vas deferens; 2 prostatic reservoirs, prostates comprising bilateral groups of cells lying dorsally in anterior trunk. Cirrus comprising a base with several large flanges, coiled tube with counterclockwise rings (see Kritsky et al., 1985, for definition); accessory piece articulating to cirrus base. Common genital pore dextroventral at level of intestinal bifurcation; muscle-like fibers attaching genital atrium to lateral margin of trunk. Seminal receptacle lying immediately anterior to ovary, comprising 2 distinct parts, distal one-third with internal lamellae; vagina sinistral. Vitellaria coextensive with gut, well developed; transverse vitelline duct immediately anterior to seminal receptacle. Disc-shaped haptor armed with ventral pair of anchors, ventral bar, 7 pairs of hooks; 6 pairs of hooks marginal, lying dorsal to haptoral disc, directed ventrally; 1 pair lying ventrally between anchor shafts; hooks with dilated shanks. Parasites of nasal cavities of characid fishes.

**Type species, host, locality:** *Rhinonastes pseudocapsaloideum* n. sp. from *Prochilodus nigricans* Agassiz (Prochilodontidae), Janaúacá Lake near Manaus, Amazonas, Brazil.

**Etymology:** The generic name is from Greek (*rhin/o* = nose; *nastes* = an occupant).

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FIGURES 1-5. *Rhinonastes pseudocapsaloideum* n. gen., n. sp. 1. Ventral view of holotype. 2. Ventral bar. 3. Hook. 4. Anchor. 5. Copulatory complex.

***Rhinonastes pseudocapsaloideum* n. sp.**

(Figs. 1-5)

**Description:** Body 514 (461-551) long, foliiform, robust, tapering anteriorly; greatest width 253 (244-269) in posterior trunk. Cephalic lobes poorly developed, 2 terminal, 2 bilateral; head organs comprising 3 groups in cephalic area, variable in number; cephalic glands elongate ovate. Eyes 4, equidistant, each with lens; members of posterior pair larger than those of anterior

pair; eyespot granules small, variable in shape. Pharynx subovate, 38 (36-40) wide; esophagus short. Peduncle tapering rapidly, attaching centrally to dorsal surface of subcircular haptor; haptor 249 (236-262) long, 236 (226-251) wide. Anchor 152 (141-168) long, lacking deep root, with superficial root, large subtriangular base, recurved point, short rod-shaped structure arising from base of point; anchor base 67 (55-89) wide. Ventral bar 61 (56-75) wide, 117 (104-120) long, with 2 sub-



medial anterior projections, anteromedial cleft, posteromedial telescoping projection, enlarged ends. Hooks similar, each 22 (21–24) long, with delicate point, erect truncate thumb, shank inflated along proximal two-thirds; FH loop one-third shank length. Cirrus with about 1½ rings; base with 3 conspicuous flanges; cirral length 190–191, proximal ring diameter 32 (29–35). Accessory piece 47 (40–51) long, rod-shaped, proximally serrated. Testis constricted medially (by ovary), 92 (79–104) long, 95 (82–109) wide; vas deferens arising from anteromedial margin of testis dorsal to ovary; prostatic reservoirs pyriform or fusiform. Ovary 98 (97–101) long, 30–31 wide; oviduct short; ootype not observed; uterus delicate; genital pore with thick lateral wall; vagina comprising distal bulbous vesicle, proximal delicate sclerotized tube; seminal receptacle elongate fusiform; vitellaria dense, coextensive with ceca.

*Specimens studied:* Holotype, INPA PA308; paratypes (4), USNM 79856, HWML 20766.

*Etymology:* The specific name refers to the superficial resemblance of the species to some members of the Capsaloidea (Monogenea).

## DISCUSSION

The general morphology of the internal organ systems of *Rhinonastes pseudocapsaloideum* justifies placement of the species in the Dactylogyridae. Presence of head organs, cephalic glands, a single testis, a vas deferens looping the left intestinal cecum, a copulatory complex comprising a cirrus and accessory piece, and confluent intestinal ceca are generally used to assign members to this family. Characters that distinguish *Rhinonastes* from all other genera in the family include presence of a dextroventral genital pore, a bilobed testis, and a ventral C-shaped ovary lying between the 2 testicular lobes. The genus is placed provisionally in the Ancyrocephalinae based on the anchor–bar complex being ventral in the haptor, presence of 7 pairs of hooks, and absence of 4A hooks.

*Rhinonastes pseudocapsaloideum* possesses several unusual haptoral features that are relatively rare and scattered among species of Dactylogyridae. Disc-shaped haptors occur in genera representing at least 4 dactylogyrid subfamilies, e.g., *Anonchohaptor* Mueller, 1938 (Pseudomuraytreminae), *Anacanthoroides* Kritsky and Thatcher, 1976 (Anacanthorinae), *Neocalceostoma* Tripathi, 1957 (Calceostomatinae), and *Anchoradiscus* Mizelle, 1941 (Ancyrocephalinae). This suggests that disc-shaped haptors of some dactylogyrids are derived features that evolved independently.

Presence of a single anchor–bar complex located ventral in the haptor is another unusual feature of *Rhinonastes*. The character also occurs

in species of *Trinigyrus* Hanek, Molnar, and Fernando, 1974, *Pseudodactylogyrus* Gussev, 1965, and *Pseudodactylogyroides* Ogawa, 1986. Kritsky et al. (1986) suggest that *Trinigyrus* is most closely related to the ancyrocephaline genus *Hamatopeduncularia* Yamaguti, 1953, based on common haptoral features. However, all *Hamatopeduncularia* possess ventral and dorsal anchor–bar complexes, and no evidence is available to suggest a close evolutionary relationship between these genera and *Rhinonastes*. Relationships of *Pseudodactylogyrus* and *Pseudodactylogyroides* are unclear. Gussev's (1965) conditional placement of *Pseudodactylogyrus* in the Dactylogyridae is based on the presence of a single anchor–bar complex. LeBrun et al. (1986) consider the genus a member of the Pseudodactylogyridae. The latter authors' rationale for proposal of Pseudodactylogyridae is based on derived larval chaetotaxy and on the presence and development of the ventral pair of anchors in larval and adult forms. Ogawa (1986) proposed Pseudodactylogyridae in the Ancyrocephalidae for *Pseudodactylogyrus* and *Pseudodactylogyroides*, because remnants of the dorsal anchor pairs represented by "needles" were present in members of *Pseudodactylogyroides*. Utilizing the presence of a ventral anchor–bar complex as criteria for assignment, the Pseudodactylogyridae and/or Pseudodactylogyridae would necessarily include *Trinigyrus* and *Rhinonastes* resulting in an unnatural or polyphyletic assemblage.

Another unusual feature of *Rhinonastes pseudocapsaloideum* is the distribution of hooks in the haptor. In this species, 6 pairs of hooks are marginal and 1 pair ventral. This arrangement differs significantly from that usually occurring in adult Dactylogyridae (see Mizelle, 1936). Indeed, species of all dactylogyrid genera with disc-shaped haptors exhibit modified hook distributions, with those of *Anonchohaptor* and *Anacanthoroides* most similar to *Rhinonastes*.

Proposal of a new family and/or subfamily for *Rhinonastes* is tempting. However, evolutionary relationships of the genus are unclear, even though *Rhinonastes* apparently shares many derived characters with various dactylogyroid taxa. The proposal of new higher taxa (subfamilies and above) should be based not only on a set of unique characters but also on features that provide information on the evolutionary relationships of the new and existing taxa. Because such studies are not within the scope of this paper, we con-

sider our assignment of the genus to the Ancyrocephalinae as provisional until analysis of these characters is made.

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## ULTRASTRUCTURAL MORPHOLOGY AND STAINING CHARACTERISTICS OF *PNEUMOCYSTIS CARINII* IN SITU AND FROM BRONCHOALVEOLAR LAVAGE

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**ABSTRACT:** The ultrastructure of *Pneumocystis carinii* obtained from rats by bronchoalveolar lavage (BAL) was compared with organisms *in situ*. All developmental forms of the organism as seen *in situ* were present in the lavage fluid. Trophozoites *in situ* were adhered to type I epithelium, had smooth surfaces, and were interdigitated with the underlying epithelium. Nonadherent trophozoites *in situ* and trophozoites in lavage fluid were more pleomorphic and irregular in shape with tubular projections extending from all surfaces. Microtubular and nuclear details not reported elsewhere were observed. To enhance the ultrastructural detail of *P. carinii* obtained by lavage, phosphotungstic and tannic acid fixation, uranyl acetate en bloc staining, and acid phosphatase staining were performed. These techniques enhanced the visibility of membranes, mitochondria, nuclei, and vacuoles. With tannic acid, increased contrast of the organism's cell coat was obtained and differences in staining intensity and thickness related to developmental stages were observed. In lavage samples with few pneumocystis organisms or those specimens heavily contaminated with macrophages, erythrocytes, or other cellular debris, tannic acid allows for easier recognition as other lung materials do not show the same distinctive staining reaction. Lung sections observed after BAL showed intact but damaged epithelial surfaces devoid of organisms. No intracellular organisms were observed. BAL removes organisms from the alveolar lumen as well as adhered organisms and is a useful method for concentrating the various morphologic forms of *P. carinii*.

*Pneumocystis carinii* (PC) is an organism capable of causing fatal pneumonia in immunosuppressed individuals. PC has recently gained prominence because of its association with acquired immune deficiency syndrome (AIDS) (Gottlieb et al., 1981; Jaffe et al., 1983; Engelberg et al., 1984). Although described by Delanoe and Delanoe in 1912, the organism and its pathobiology are still poorly understood and even its taxonomic position, i.e., fungus or protozoa, is uncertain (Frenkel et al., 1966; Vavra and Kucera, 1970; Ham et al., 1971; Yoneda et al., 1982).

Numerous transmission electron microscopic (TEM) studies of the ultrastructure of PC have been reported. The majority of these describe the organism as seen *in situ* (Vavra and Kucera, 1970; Ham et al., 1971; Campbell, 1972; Vossen et al., 1978; Lanken et al., 1980; Hasleton et al., 1981; Yoneda and Walzer, 1981; Yoneda et al., 1982; Yoshida et al., 1984). The ultrastructure of PC as harvested from bronchoalveolar lavage (BAL) was of interest for several reasons: to establish if all forms are recovered, to determine if morphological differences from *in situ* forms occur, and to further elucidate the fine structure. The potential airborne mode of transmission of PC makes an understanding of the contents of BAL

essential (Hendley and Weller, 1971; Hughes, 1982; Furata et al., 1984). Furthermore, diagnostic procedures commonly employ BAL to rule out PC (Drew et al., 1974; Ognibene et al., 1984; Hartman et al., 1985; Springmeyer et al., 1986). A few ultrastructural studies of PC in lung homogenates (Walzer et al., 1979) or cyst concentrates (Ikai et al., 1977) have been reported. In this study the ultrastructural morphology of PC obtained from rat BAL is reported and compared with *in situ* specimens. In addition, techniques to enhance the ultrastructural detail of BAL-obtained organisms are reported.

### MATERIALS AND METHODS

Forty-eight Sprague-Dawley male rats, 200-250 g, were used as the host animals. Pneumocystis pneumonia was induced by subcutaneous injection of cortisone acetate (25 mg/dose 2×/wk). Animals were fed a low protein diet (8%) and provided drinking water (*ad libitum*) containing 1 mg/ml tetracycline to control bacterial infections (Frenkel et al., 1966). After 8-10 wk of cortisone treatment, rats were anesthetized by intramuscular injection with a 10:1 mixture of ketamine hydrochloride (Vetalar, Parke Davis) and acepromazine maleate (Aveco Co.) 0.1 ml/100 g body weight. BAL cells were harvested with 50 ml of warm Hanks' balanced salt solution (HBSS). Five-ml aliquots of HBSS were injected into the lungs and suctioned by way of a tracheotomy. The lavage fluid was centrifuged at 30 g for 5 min. The resulting cell pellet contained most of the alveolar macrophages and some cysts and trophozoites. The supernatant was then centrifuged at 1,300 g for 30 min (Masur and Jones, 1978). This pellet

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contained most of the organisms. Both pellets were washed 3 times in HBSS and fixed within 1 hr as described below.

Six animals not subjected to BAL were euthanized, the lungs removed, and sections cut into 1-mm<sup>3</sup> tissue blocks while immersed in cold 5% glutaraldehyde (GA) buffered with 0.1 M cacodylate (Cdy) (pH 7.2). During the first hour of fixation, lung tissue was degassed in a vacuum chamber at ambient temperature. Tissues were subsequently postfixed in 1% OsO<sub>4</sub> for 1 hr (4 C), dehydrated in a graded ethanol (EtOH) series, and embedded in araldite. Thin sections (60 nm) were poststained with uranyl acetate (UA) and lead citrate.

### Staining procedures

*Uranyl acetate en bloc stain* (Hayat, 1981): BAL specimens were fixed overnight in 5% GA (4 C), postfixed with OsO<sub>4</sub> for 1 hr (4 C), rinsed with 50% EtOH, followed by 15 min incubation in 2% aqueous UA prepared in 50% EtOH. Specimens were subsequently dehydrated and embedded as stated above for lung blocks.

*Tannic acid* (Hayat, 1981): Tannic acid (4%) in 2.5% GA buffered with 0.1 M Cdy was prepared immediately before use and adjusted with dilute NaOH to either a pH of 7.2 or 6.8. After overnight fixation at 4 C, specimens were postfixed with OsO<sub>4</sub> and processed as stated above.

*Phosphotungstic acid (PTA) en bloc stain* (Dawes, 1979): Specimens were fixed in 5% GA overnight (4 C), dehydrated in a graded EtOH series, and incubated for 1 hr in 2% PTA dissolved in absolute EtOH. Samples were then rinsed twice in propylene oxide (PPO) (4 C), followed by 2 changes of PPO mixed in 0.01% NaOH. Final embedding was in araldite. Sections were poststained with UA and lead acetate.

*Acid phosphatase, modified Gomori technique* (Barka and Anderson, 1962): BAL cell pellets were fixed in 0.5% GA in 0.1 M Cdy buffer for 5 min at ambient temperature. Cell pellets were then transferred into 0.1 M Cdy buffer before incubation for enzyme reaction. Negative control specimens were incubated in the absence of substrate.

Specimens from at least 3 different animals were utilized for each of the above staining procedures.

## RESULTS

### *Pneumocystis carinii* in situ

Observations of PC *in situ* were similar to those reported previously (Bommer, 1964; Vavra and Kucera, 1970; Ham et al., 1971; Campbell, 1972; Vossen et al., 1978; Hasleton et al., 1981; Yoneda and Walzer, 1981; Yoneda et al., 1982; Henshaw et al., 1984; Yoshida et al., 1984). Trophozoites and cyst forms were associated with alveolar epithelial surfaces. In some areas the organisms formed multilayers and extended into the alveolar lumens, occasionally completely occluding the alveoli (Fig. 1).

PC trophozoites *in situ* were closely apposed to type I pneumocytes but no attachment was

observed with nearby secretory type II pneumocytes (Fig. 1). The overlying trophozoites formed complicated infoldings and interdigitations with the type I pneumocytes (Fig. 2) (Henshaw et al., 1984; Long et al., 1986). Thin finger-like projections of epithelium were observed to extend between organisms and even partially envelope luminal surfaces of the attached PC (Figs. 2, 3). Developing cysts were also adherent and in some cases were partially enveloped by type I epithelium (Fig. 4).

Adherent trophozoites, unlike those seen in BAL, were plump and rounded. The trophozoite cell wall had smooth contours with few of the distinctive tubular surface projections. The few tubular projections associated with adhered trophozoites were located on the luminal surfaces of the organism. Trophozoites with surface projections on all sides were not adherent to the type I pneumocytes (Fig. 3).

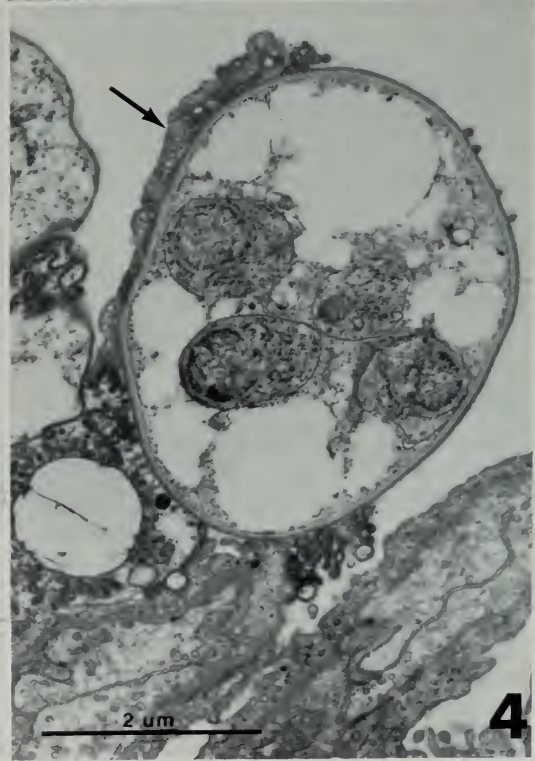
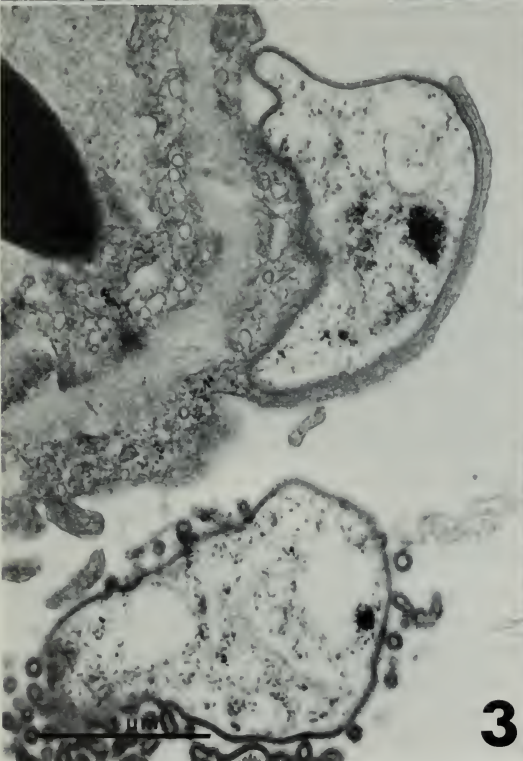
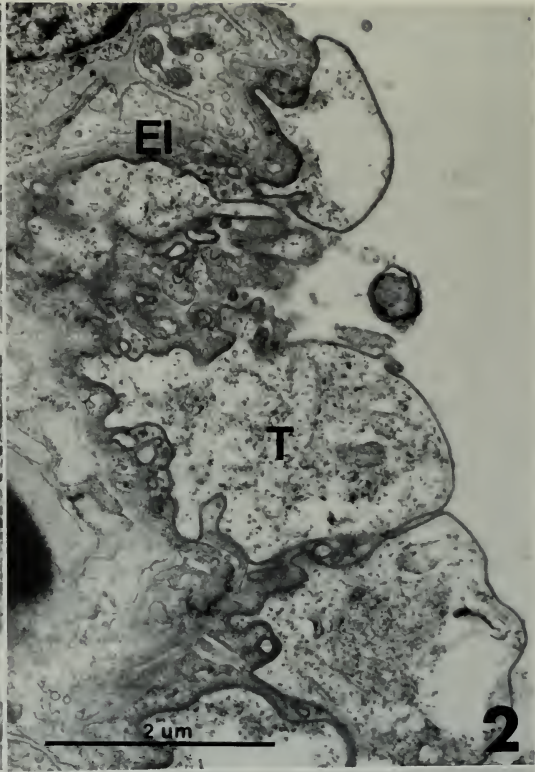
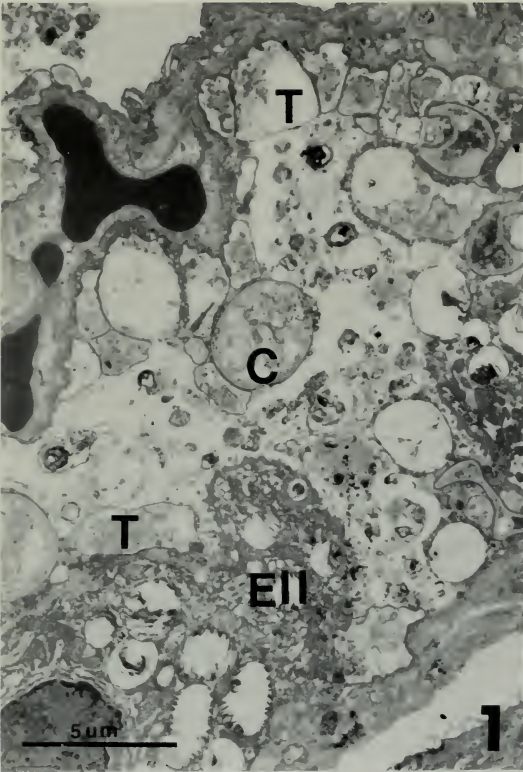
Types I and II epithelial cells of normal rat alveoli processed for TEM after BAL remained intact and appeared normal with secretory products in type II cells and junctional complexes between types I and II. Discrete pinocytotic vesicles were present in both type I epithelial cells and endothelial cells (Fig. 5). Lung tissue from 4 cortisone-treated rats was processed for TEM after BAL. Sections from 2 blocks of each lung sample were scanned. Three of the rats were negative for residual, adherent PC. In the fourth rat, a few trophozoites were observed in sections from one of the blocks. PC-infected lung processed after BAL showed a more irregular, edematous surface. Type I epithelium appeared hypertrophic and less dense with fewer and more irregular-shaped vacuoles and vesicles. The underlying basement lamina was greatly thickened. Despite changes in morphology, the surface epithelium was still intact and largely devoid of adherent trophozoites (Fig. 6). PC was not observed intracellularly in rat lung sections with or without BAL.

### *Pneumocystis carinii* in BAL fluid

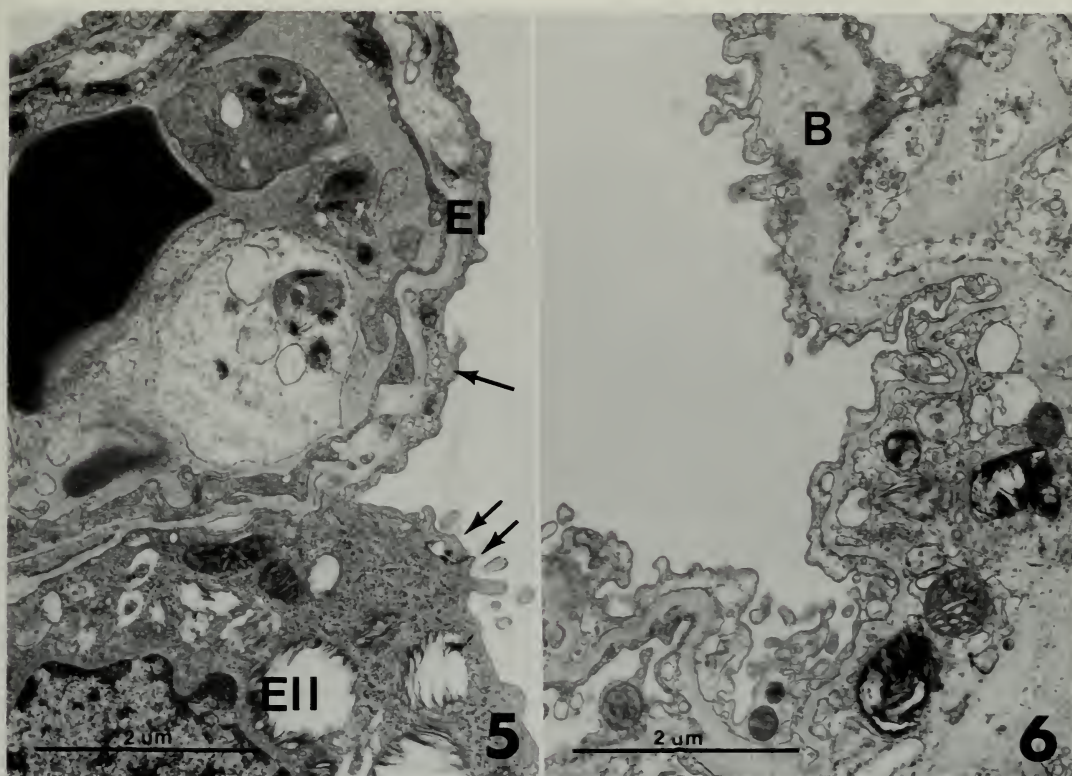
All previously reported forms of PC found *in situ* were present in BAL fluids. Trophozoites, empty and collapsed cysts, as well as cysts containing characteristic intracystic bodies, and cysts containing small trophozoites were observed (Figs. 7–14).

Trophozoites obtained from BAL were similar to nonadherent trophozoites observed *in situ*.









FIGURES 5, 6. Electron micrographs of respiratory epithelium after BAL. 5. TEM of normal respiratory epithelium after BAL. Type I (EI) and type II (EII) epithelia are indicated. An erythrocyte (RBC) and portions of leukocytes are present within the capillary lumen. Single arrow indicates discrete pinocytotic vesicles and double arrow indicates a junctional complex between type I and type II pneumocytes. 6. PC-infected respiratory epithelium after BAL. The basal lamina (B) is thickened and underlies edematous, irregular type I pneumocytes.

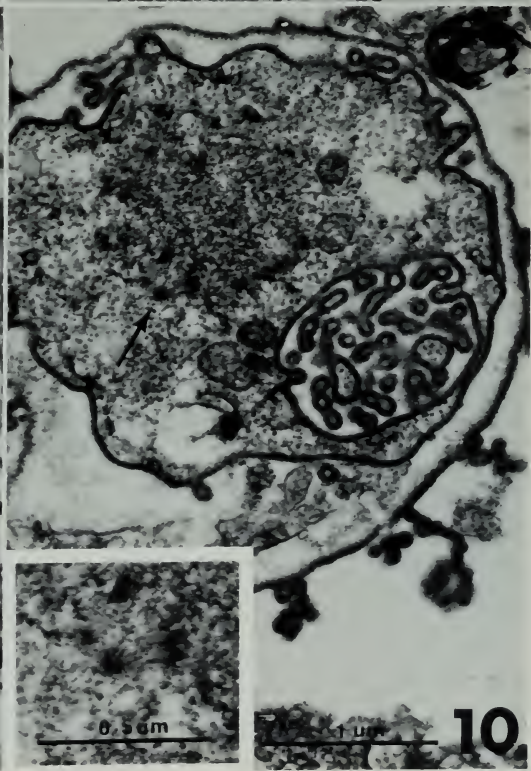
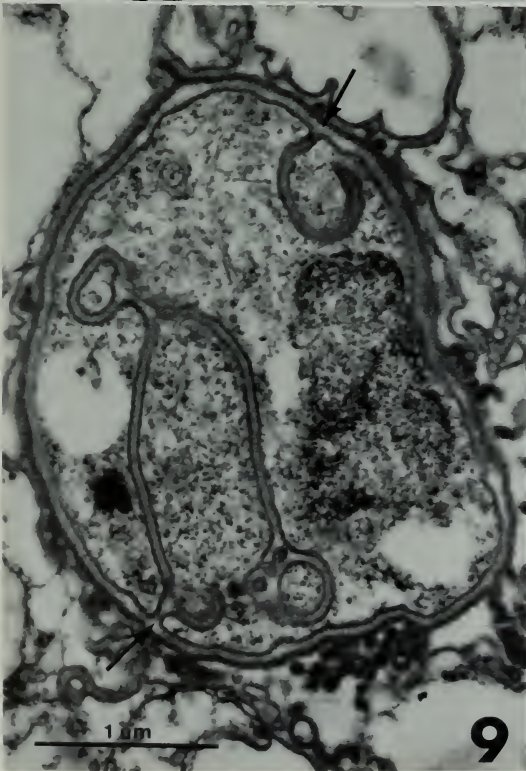
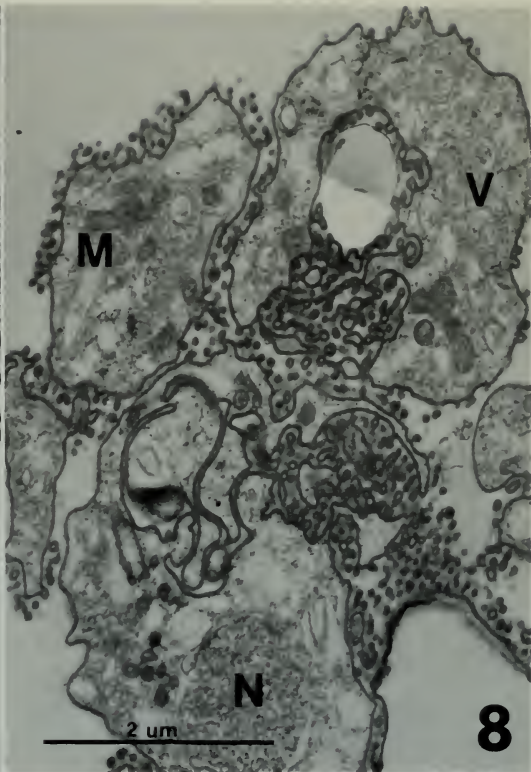
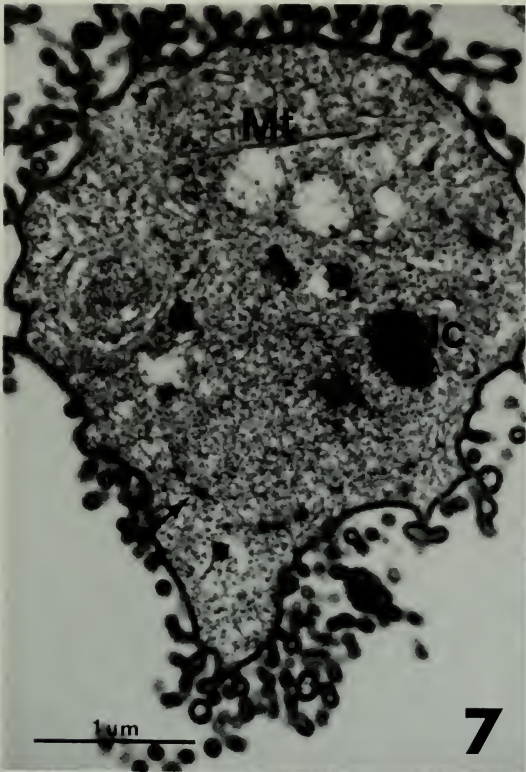
BAL trophozoites were characterized by more pleomorphic, irregular shapes and an increase in the number of tubular projections. The cell cytoplasm and accompanying cell membrane extended into the tubular projections. These projections branched dichotomously and extended in various directions (Fig. 7). Aggregates of PC with their tubular projections intermeshed were seen in BAL samples (Fig. 8). More aggregates were observed with the use of HBSS containing  $\text{Ca}^{+2}$  and  $\text{Mg}^{+2}$ , whereas fewer were observed in the absence of  $\text{Ca}^{+2}$  and  $\text{Mg}^{+2}$ . Therefore, aggregate formation may be a calcium-dependent process. Similar observations were made by Gradus and Ivey (1986).

The average widths and lengths of trophozoites in BAL and *in situ* were determined. Equal numbers of randomly selected trophozoites were measured. The average widths for trophozoites in BAL were  $1.75 (\pm 0.93) \mu\text{m}$ , with lengths measuring  $2.83 (\pm 0.83) \mu\text{m}$ . Trophozoites *in situ* measured  $1.83 (\pm 0.83) \mu\text{m} \times 2.9 (\pm 1.26) \mu\text{m}$  with an  $n = 30$ . Therefore there was no significant difference in size. Both BAL and *in situ* trophozoites were more ovoid in shape. More variation in length occurred among adherent trophozoites. Length was not necessarily associated with the adherent surface.

Cytoplasmic microtubules were occasionally observed to extend into pseudopodia of the tro-

FIGURES 1–4. Electron micrographs of *Pneumocystis carinii* *in vivo*. 1. Alveolus occluded by cell debris and surfactant granules. Note trophozoites (T) and cysts (C) adherent to type I epithelium but not type II (EII). 2. Interdigitations formed between type I epithelium (EI) and trophozoites (T). 3. Adherent trophozoites partially enveloped by a finger-like projection of cytoplasm from type I pneumocyte. Note the pinocytotic vesicles of the endothelium. An unattached trophozoite with multiple tubular extensions is in close proximity. 4. *Pneumocystis carinii* cyst partially enveloped by type I pneumocyte (arrow).







phozoites and were anchored to the cell membrane. Microtubules were also associated with mitochondria and the nuclear membranes.

A distinctive nuclear structure was noted in BAL-obtained trophozoites. Finely dispersed nuclear chromatin without a delimiting nuclear membrane could be observed distinct from the ground substance of the cytoplasm. Arranged in regular intervals along the nuclear periphery, occasional arcuate condensations were seen (Figs. 7, 10, 14). Radiating filaments extended from these condensations (Fig. 10, inset). Single nucleoli were noted (Fig. 7).

Figures 9 and 10 are examples of an unusual form of trophozoite observed in BAL specimens. *Pneumocystis carinii* trophozoites are typically enclosed by a cell membrane and a characteristically thin, but densely stained cell coat or pellicle of 20–30 nm thickness. The trophozoites in Figures 9 and 10 appear to be mature trophozoites contained within another cell wall. Maturity of the trophozoites is shown by their size and irregular shape, presence of cell membranes, and characteristic cell coats (Vavra and Kucera, 1970; Vossen et al., 1978; Matsumoto and Yoshida, 1984). In addition, these trophozoites form typical tubular projections. Serial sections of the organism in Figure 9 reveal that the organism is capable of forming deep folds or invaginations of the cell membrane and cell wall.

Cyst forms were identified by their thickened cell walls and more regular ovoid shapes. Tubular projections were present on the cell wall of cysts but were fewer in number than on trophozoites. These projections were also shorter and no longer continuous with the cell cytoplasm and internal cell membrane. Cysts containing characteristic intracystic bodies (Fig. 13) as well as cysts containing small trophozoites (Fig. 14) were observed. Trophozoites resembling intracystic bodies but not enclosed within a cyst were occasionally seen.

Figure 11 is a micrograph of a developing cyst. It has a regular, ovoid shape and a barely dis-

cernible triple-layered wall. Of particular interest is the presence of a plaque-like spindle pole in the cytoplasm of the cyst. Astral microtubules, as well as polar and chromosomal microtubules with their associated dense chromatin masses, can be seen emanating from the spindle plaque. Serial sections confirmed the spindle plaque to be a microtubule organizing center. Microtubules extending away from the chromatin masses were seen to converge toward the opposite end of the precyst and perhaps to another spindle plaque located out of the plane of section. The spindle plaque consists of 2 parts: a slightly curved outer cap composed of a dense rim with a lighter center, and an inner face that consists of a linear structure from which evenly spaced knobs continuous with the microtubules project.

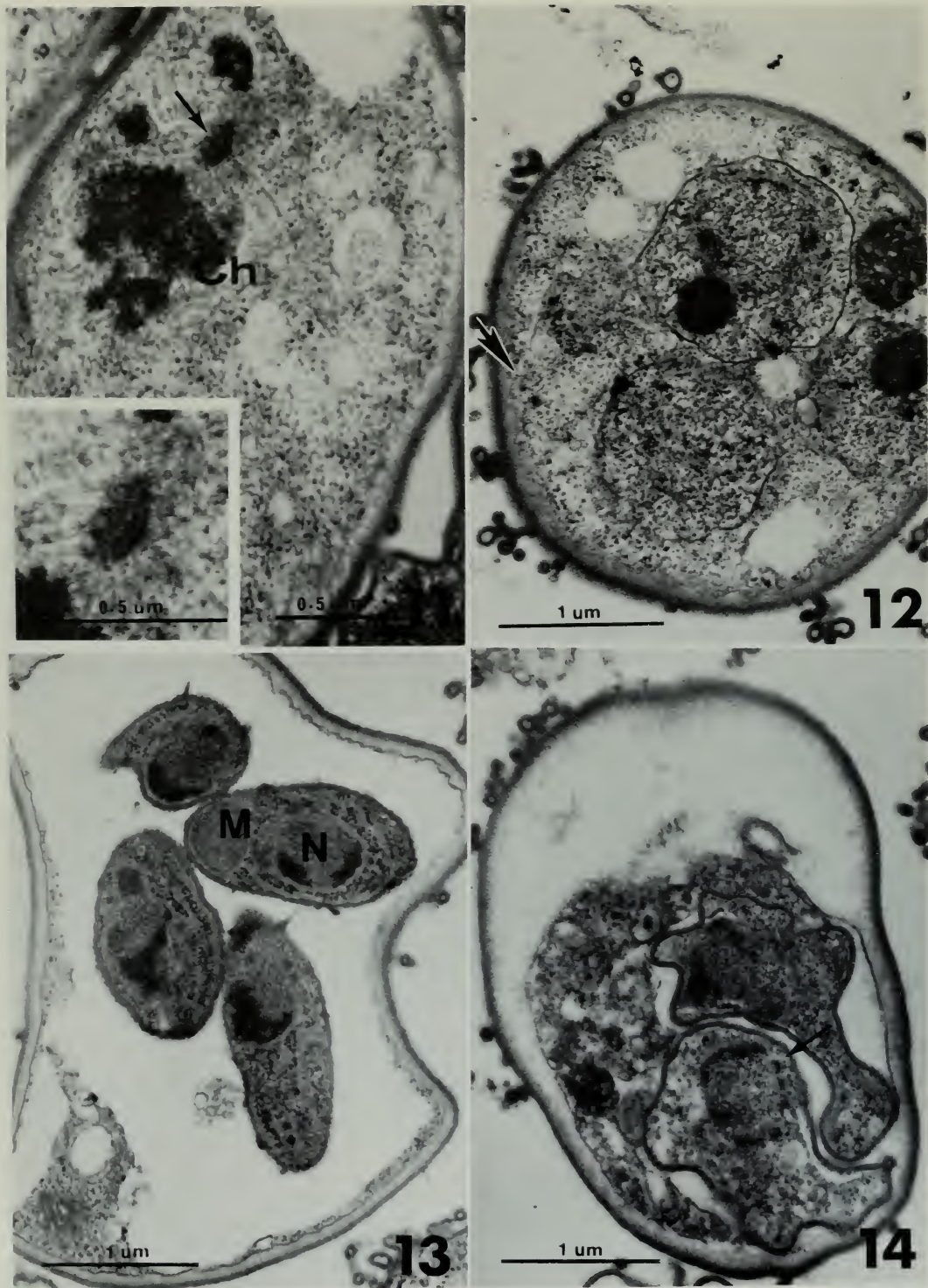
In a further developed cyst (Fig. 12), intracystic bodies are partially delimited with a plasma membrane. Contained within each developing intracystic body are a mitochondrion and a finely vesiculated nucleus. A spindle with radiating microtubules is present at the extreme periphery of the cyst and in fact seems to be embedded within the cell wall. The cell wall at this stage also seems to be thicker, with a much wider electron-lucent layer than the cyst seen in Figure 11. Upon further maturation, the nucleus of the intracystic bodies condenses and the heterochromatin associates with definitive nuclear membranes (Fig. 13). A single mitochondrion is present, with the rest of the cytoplasm filled with organized arrays of rough endoplasmic reticulum.

#### PTA staining

PTA staining revealed intracellular membranes as negative images (Fig. 15). A negative-staining plasma membrane beneath a heavily stained cell coat was observed. This membrane extended into the tubular surface projections. An intact double nuclear envelope enclosed dark-staining nuclear material. The nucleus was irregular in shape. Numerous mitochondria in various spatial configurations were observed. The

FIGURES 7–10. TEM's of trophozoites seen in BAL. 7. Trophozoite with multiple, branching tubular projections. Microtubules (Mt), arcuate condensations around vesicular nucleus (arrow), and nucleolus (Nc) are present. 8. Aggregate of pleomorphic trophozoites with intermeshed projections. HBSS containing  $\text{Ca}^{+2}$  and  $\text{Mg}^{+2}$  was used; stained with en bloc UA. Many mitochondria (M), membrane-bound vacuoles (V), and a nucleus (N) are present. 9. Mature trophozoite completely enclosed within a thin wall. This is a representative serial section showing deep folds or invaginations of the trophozoite cell wall (arrows). 10. Trophozoite within a thin-walled mother cell. Note nuclear condensations (arrow). Inset is a higher magnification of arcuate condensation and filament configuration. Honeycomb-appearing structure to the right represents multiple pseudopodia projecting into a partially formed vesicle or invagination.





FIGURES 11-14. Cyst forms of *P. carinii* from BAL arranged according to maturational stage. 11. Precyst with spindle plaque. Inset is a higher magnification showing radiating microtubules and associated dense chromatin masses (Ch). 12. Further developed cyst with partially delimited intracystic bodies. A spindle formation

mitochondrial matrix was dense in contrast to the negative image of the cristae. Membrane-bound vacuoles and electron-lucent areas in the cytoplasm of trophozoites, which appear empty with regular TEM processing, were stained with PTA (Fig. 16).

Tannic acid fixation greatly enhanced staining of the pneumocystis cell wall (Fig. 17). Fixation at pH 6.85 provided optimal preservation of internal structures. Tannic acid evidently will penetrate the cell wall of cysts and reacts with the wall material of developing trophozoites within cysts. However, the thinner, fuzzy cell coat of intracystic bodies did not react with tannic acid. The reaction of tannic acid with PC wall material appears to be specific, because macrophages, erythrocytes, and tubular myelin, also present in BAL, were negative (Fig. 17).

#### Acid phosphatase staining

The inset of Figure 18 shows a portion of the peripheral cytoplasm of an alveolar macrophage obtained by BAL. The plasma membrane extends into numerous pseudopodia as seen on the upper right. The plasma membrane and other intracellular membranes are unstained as no counterstain is used in this procedure. Numerous acid phosphatase-staining lysosomes are present as evidenced by the dark-staining precipitate within membrane-bound vesicles.

In Figure 18 the outline of a trophozoite is clearly seen because of deposition of acid phosphatase lead reaction product in the cell wall of the organism. A similar reaction was noted by Barton and Campbell (1969).

#### DISCUSSION

Previous ultrastructural studies have been concerned primarily with the morphology of PC *in situ*. However, as one mode of transmission may be airborne, and thus likely from respiratory secretions (Hendley and Weller, 1971; Hughes, 1982; Furata et al., 1984), and because clinical diagnostic procedures utilize BAL, it was important to determine the ultrastructural morphology of PC in BAL samples. The limitations of light microscopy have been shown in determining the presence of the trophozoite form

(Milder et al., 1980), which is the predominant form in early infection and has been reported to persist after treatment (Lanken et al., 1980; El-Sadr and Sidhu, 1986). The BAL technique and TEM processing utilized in this study yielded a concentrate of all previously reported forms of PC with ultrastructural morphology preserved intact.

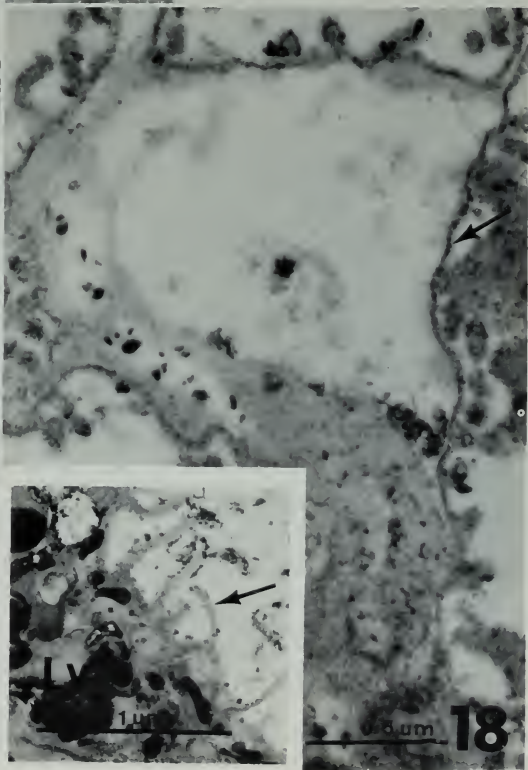
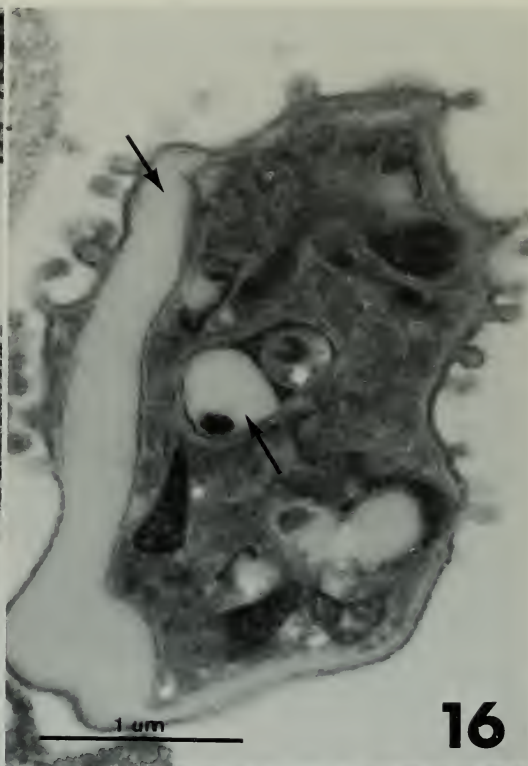
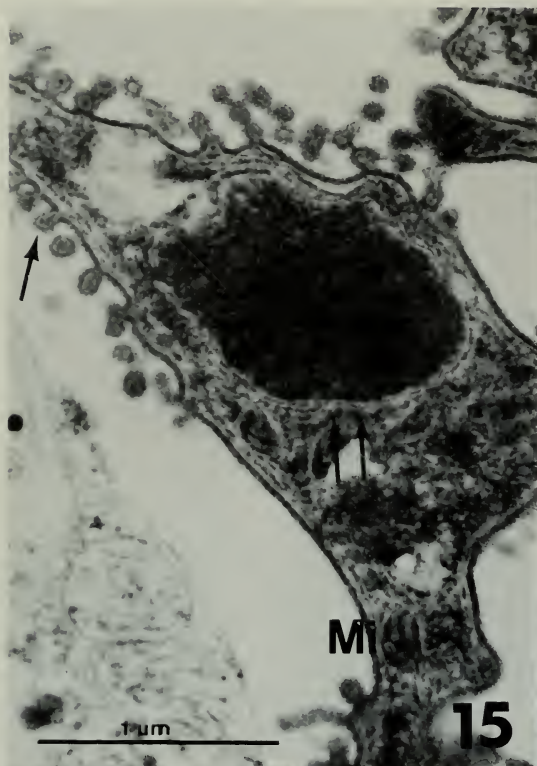
Trophozoites in BAL were extremely pleomorphic and had numerous tubular projections over their surfaces. The plasma membrane was seen to extend into the projections; therefore, these are considered to be evaginations of the organism itself, not just the cell wall. *In situ*, adherent organisms were observed to interdigitate with the alveolar epithelium and to have a more plump, spherical morphology with few tubular projections when compared with BAL samples. Adherent organisms were only associated with type I pneumocytes and had projections only on their luminal surfaces. These findings are in agreement with other authors who have studied PC in TEM thin sections and freeze-fracture preparations (Yoneda and Walzer, 1981; Henshaw et al., 1984; Yoshida et al., 1984; Long et al., 1986). Thus, we would agree with those authors who consider that the tubular projections are not used for attachment purposes, and would propose that they may act as sensors for suitable environments and/or provide increased surface area for nutrient and gas exchange. Attachment also establishes a definite polarity to the trophozoite, which may be necessary for growth and development. Lack of attachment may be a factor that contributes to the pleomorphism of BAL trophozoites.

Type II epithelial cells have highly irregular luminal surfaces, are actively secreting cells, and may lack a receptor for PC adherence. Type I cells, however, have smooth surfaces, are capable of forming complex membrane interdigitations, and may have PC surface receptors. Partial embedment of the organism in type I pneumocytes and infolding of the epithelium around the organism may not only provide protection to the organism but also increased surface area for nutritional absorbance. Long et al. (1986) suggested that the trophozoites form the interdigitations in

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(arrow) is seen at the periphery of the cyst. 13. Cyst with fully developed intracystic bodies. Note thin cell coat associated with cell membrane of intracystic bodies. A large mitochondrion (M), well-defined nucleus (N), and rough endoplasmic reticulum are evident. 14. Maturing trophozoites contained within a cyst. Note arcuate heterochromatin condensations (arrow).





order to anchor themselves to the epithelium. Observations of lung tissue after BAL show alveolar surfaces to be vesiculated with some bleb formations but essentially intact and devoid of organisms (Fig. 6). Therefore, attachment may be a reversible process, as organisms can be washed from the alveolar epithelial surface or adherent material used for attachment purposes may be dissolved. No morphological membrane specializations for attachment were observed. Adherence of cysts to type I epithelium was also noted and similar advantages as mentioned for trophozoites would apply equally well to the cyst form.

Type I epithelium of infected rats after BAL showed a more irregular edematous surface when compared to lung samples from untreated control rats subjected to BAL. A plausible explanation for these changes is interaction of organisms with the underlying epithelium. The epithelium forms invaginations with the organism resulting in increased surface area. The organism is probably an irritant and causes production of a thickened basal lamina. Gas and fluid exchange is impeded with establishment of PC infection and contributes to hypertrophy and loss of vacuoles and vesicles involved in normal transport. We also noted hyperplasia of type II cells (Gottschall et al., 1979), which would contribute to increased surfactant production and further changes in the fluid environment of the alveoli. With severe prolonged infection, focal necrosis of type I cells occurs (Lanken et al., 1980; Yoneda and Walzer, 1981).

Tubular projections were present in smaller numbers on cyst forms but were no longer continuous with the cell cytoplasm and therefore are perhaps superfluous and nonfunctional. Yoshikawa and Yoshida (1986), using freeze-fracture techniques, described tubular projections connected with the outer granular layer of precysts and cysts. They considered these to be fragments that probably result from self cutting of the tu-

bular expansions during development from trophozoite to cyst.

To enhance the appearance of intracellular structures, PTA was employed. PTA revealed membranes as negative images, and trophozoites with well-defined nuclear envelopes were observed. PTA has been shown to have affinity for basic proteins associated with DNA and nucleolar material and heavy, uniform nuclear staining was obtained in this study. The cristae of mitochondria and variably shaped, membrane-bound vacuoles were easily identified. Large electron-lucent areas, as seen with conventional TEM fixation and staining procedures, were stained by PTA (Figs. 15, 16).

Heavy staining of the cell wall was observed with PTA and tannic acid. We believe this staining is related to the presence of polysaccharides and glycoproteins in the cell wall because BAL samples were washed multiple times and therefore glycoproteins associated with lung tissue should be diminished. Ruthenium red and periodic acid Schiff have affinities for mucopolysaccharides and have been observed to stain PC *in situ* (Bommer, 1964; Yoneda and Walzer, 1981).

Tannic acid has been used to enhance the electron density of a variety of cellular structures (Simionescu and Simionescu, 1976a; Saito et al., 1978) and acts primarily as a mordant between osmium-treated structures and lead. It may also act to stabilize tissue components and prevent extraction during dehydration and subsequent processing of tissues (Simionescu and Simionescu, 1976a, 1976b). Yoshida et al. (1984) used tannic acid for *in situ* observation of the alveolar lining layer, but did not observe differential staining of PC from other lung constituents. We used a similarly prepared tannic acid fixative. Yoshida perfused fixative through the right ventricle and infused fixative through the trachea to preserve the surface film of the alveolar lining. Glycoproteins from alveolar secretions were

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FIGURES 15–18. Ultrastructural staining characteristics of *P. carinii*. 15. PTA-stained trophozoite revealing negative images of membranes. Plasma membrane extending into tubular projections is indicated by a single arrow. A nuclear envelope completely encloses an intensely stained nucleus (double arrow). Cristae of mitochondria (Mi) are also evident. 16. PTA-stained trophozoite. Note membrane-bound vacuoles filled with light-staining material (arrows). 17. Tannic acid-fixed trophozoites and developing trophozoites within a cyst (C). Note heavily stained cell wall of the cyst as well as the cell coat of mature trophozoites (T) and trophozoites within the cyst. Tubular myelin (TM) is not stained. 18. Trophozoite stained for the presence of acid phosphatase. Specimens were not counterstained. Note deposition of lead reaction product along cell coat of organism (arrow). Inset shows a portion of the peripheral cytoplasm of an alveolar macrophage. Lysosomes (Ly) are heavily stained for acid phosphatase. Note negative staining of plasma membrane extended into filopodia (arrow).



therefore fixed and precipitated onto PC and other cells lining the alveoli. Its use with BAL preparations resulted in greatly enhanced contrast of the cyst cell wall and produced a clear demarcation between the outer and inner layers (Fig. 17).

Trophozoites share a similar staining cell coat without the electron-lucent layer. Tannic acid fixation was efficacious in visualizing the presence of PC in BAL samples, because other cellular elements present (i.e., macrophages, erythrocytes, tubular myelin, lipids, membranous debris) were negative. Intracystic bodies, even though they did show a thin fuzzy coat, did not demonstrate tannic acid affinity. The negative reaction of the intracystic body as compared with the cell coat of the trophozoite stage and cyst implies that the cell wall constituents change with the developmental stage of the organism. Tannic acid is capable of penetrating the cell wall as developing trophozoites within cysts also show enhanced contrast.

Acid phosphatase reaction product was observed along the cell coat of the trophozoites and cysts. Barton and Campbell (1969), using a method for detecting liver acid phosphatase, also noted positive reaction product in the cell coat and nucleus of PC. Barton and Campbell's study was done exclusively on *in situ* specimens, whereas the positive staining noted here was performed on BAL-obtained organisms.

Acid phosphatase was not observed in membrane-bound vacuoles, however, other unidentified hydrolytic enzymes may be present. Acid phosphatase is considered a marker for lysosomes and the absence of acid phosphatase was interpreted by Barton and Campbell (1969) to indicate lack of phagocytic function and accompanying hydrolytic processes for nutrition. The presence of the cell coat probably inhibits phagocytosis or pinocytosis. Thus, diffusion of nutrients through the cell membrane is necessary. Presence of acid phosphatase in the cell coat may aid in the breakdown and absorbance of nutrients much like enzymes in the glycocalyx of intestinal absorptive cells.

With BAL procedures, concentration of PC is possible and developing forms in addition to the trophozoite and cyst were observed. Also, better preservation of microtubules and nuclear structures was achieved than with whole lung samples. Intact cysts containing 4 or more intracystic bodies were observed. Evidently, these intracys-

tic bodies are released, because small numbers of free, intracystic bodies outside of cysts were observed. These forms have been reported previously and are believed to become mature trophozoites (Vavra and Kucera, 1970; Vossen et al., 1978). Trophozoites within thick-walled cysts (Fig. 14) were also observed. This finding supports the above-cited authors' proposed life cycle of development of intracystic bodies within a thick-walled cyst and subsequent release of mature trophozoites.

How newly formed trophozoites are released from a cyst has not been adequately demonstrated. Although Vavra and Kucera (1970) showed a trophozoite escaping through a break in the cyst wall, a consistent mode of release has not been demonstrated. We did not observe any gaps in cyst walls. The presence of trophozoites within thin walls may indicate that not all trophozoites are released and degradation of the cyst wall must occur before release.

Rare numbers of trophozoites within thin walls were observed and may indicate the presence of a daughter trophozoite within a mother trophozoite, and thus, endogeny (Figs. 9, 10). Development of PC in thin-walled cysts is an alternate life cycle proposed by Vossen et al. (1978). An alternative explanation for the presence of trophozoites within thin walls is that the outer thin wall may result from degradation of the inner layer of the cyst wall and cyst plasmalemma. We observed cysts containing developing trophozoites. The cell walls of these cysts did not have an inner electron-lucent layer and exhibited fragments of a cyst plasmalemma.

Figure 11 was identified as an early precyst according to the characteristics listed by Matsumoto and Yoshida (1984). These authors were able to identify synaptonemal complexes in precysts and therefore establish meiosis as part of the developmental process of PC. We did not see synaptonemal complexes in our samples but did observe microtubules and spindle formations as seen in Figures 11 and 12. As in the above reference we were also unable to observe any centrioles. Matsumoto and Yoshida did observe the presence of dense bodies from which spindle microtubules radiated. As this body was also present in interphase nuclei, they called it a nucleus-associated organelle (NAO). The microtubule-associated structure we observed was not present in interphase nuclei and has much more detail than the dense body seen by Matsumoto

and Yoshida. From comparisons with micrographs of meiosis in yeast, the microtubule-associated structure resembles a spindle plaque (Peterson et al., 1972). Unlike meiosis in yeast or the NAO, the spindle plaque in PC was not associated with the nuclear envelope. At later stages of cyst development (Fig. 12), microtubule organizing centers (Pickett-Heaps, 1971) are located at the periphery of the cyst.

Other structures associated with the nucleus that have not been reported before are the arcuate condensations along the periphery of the nucleus (Figs. 7, 10, 14). These were primarily observed in trophozoites, although comparable structures were observed in occasional intracystic bodies. These condensations may be heterochromatin or, because of the regular arrangement and presence of fine filaments extending toward the center of the nucleus, may be nuclear pore structures associated with heterochromatin. In some trophozoites the condensations were associated with fragments of the nuclear envelope. Nuclear membrane was not seen consistently in trophozoites with routine EM processing, even though finely vesiculated nuclear material could be discerned to be distinct from the ground substance of the cytoplasm. This may be a problem of fixation or may represent actual stages in the developmental process of PC.

In summary, this study has shown that all forms of PC may be recovered in BAL fluid. BAL allows for concentration of PC and preservation of ultrastructural morphology. Various reproductive forms and nuclear-associated structures were found in BAL-obtained PC. The use of en bloc staining, tannic acid, and acid phosphatase greatly enhanced the ultrastructural detail of PC organisms.

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# RESEARCH NOTES

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## Effects of *Trichinella spiralis* Infections Upon Bone Marrow Stem Cells in BALB/c Mice

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**ABSTRACT:** *Trichinella spiralis* infections provoke a variety of responses in the host, some of which involve stem cell proliferation and myeloid cell maturation, increases in the mast cell precursor cell populations, and maturation and eosinopoiesis. Very little is known about the influence of *T. spiralis* upon bone marrow stem cells and splenic colony formation. In the present communication we report that *T. spiralis* infection in mice stimulates the generation of colony-forming units in the spleen (CFU-S). Passive transfer of bone marrow cells from uninfected BALB/c mice to X-irradiated (650 R) *T. spiralis*-infected recipients resulted in a significant increase of CFU-S at 14 and 24 days postinfection. Passive transfer of bone marrow cells from *T. spiralis*-infected mice to X-irradiated uninfected mice also resulted in increased numbers of CFU-S in the donor mice at 24 days postinfection. These findings strongly suggest that *T. spiralis* infection conditions the microenvironment in the spleen which stimulates CFU-S.

*Trichinella spiralis* infections provoke a wide variety of responses and changes in the host (Castro and Bullick, 1983; Stewart, 1983; Wakelin and Denham, 1983; Weatherly, 1983), and some of these responses and changes are known to be directly related to the number of stem cells present and to the amount of stem cell proliferation or maturation. For example, inflammatory reactions stimulated by infection involve accelerated stem cell proliferation and myeloid cell maturation (Bender et al., 1987). There is a single report (Parmentier et al., 1987) which indicates that mast cell precursor cells increase and mature in the bone marrow after *T. spiralis* infection. Also, infection of rats with *T. spiralis* induces significant eosinopoiesis in the bone marrow (Ismail and Tanner, 1972). There appears to be no other information demonstrating the influence of *T. spiralis* infection upon the increases in bone marrow stem cells and splenic colony formation.

In the present communication, a preliminary observation is reported which indicates that *T. spiralis* infection stimulates the formation of colony-forming units in the spleen (CFU-S).

Ten BALB/c female mice at 8-10 wk of age were used in each donor and recipient group. Bone marrow was flushed from femurs of mice with Eagle's MEM using a 23-gauge hypodermic needle plus syringe. The bone marrow was dispersed by repeated passage through the needle, washed in several changes of fresh Eagle's MEM, and the final suspension of cells was adjusted to desired concentrations. Viability of the cells was determined by measuring trypan blue exclusion. For bone marrow cell transfer,  $10^5$  cells were injected into the tail vein of mice that were previously X-irradiated (650 R). Spleens were excised from mice 9 days postinjection and fixed in Telle's medium. The CFU-S were observed as white nodules in the surfaces of each spleen and counted (Till and McCulloch, 1961). Means were determined for 10 mice in each group, and Student's *t*-test was employed for statistical analysis.

Mice were infected with 300  $L_1$  per os and killed at several time intervals postinfection (DPI; 5, 14, 24, and 42 days). Bone marrow cells were removed at these times and transferred to X-irradiated uninfected mice. Uninfected mice were also killed as above and bone marrow cells were collected and transferred to infected X-irradiated mice and to irradiated uninfected mice (controls). The transfer of bone marrow from infected mice to the irradiated uninfected mice resulted in a significant increase in CFU-S only at 24 DPI. At 42 DPI CFU-S were normal. However, the transfer of bone marrow from uninfected mice to irradiated infected mice produced CFU-S significantly in excess of that found in controls at 14 DPI and at 24 DPI as well, after which time CFU-S returned to normal (Table I). The increased number in CFU-S that was observed in the infected irradiated mice receiving bone marrow cells from uninfected mice corresponded to the same days that worms were expelled from the intestines of mice receiving primary infections (Table II).



TABLE I. Nodular counts in the surface of spleens in BALB/c mice.

Days post-infection	Bone marrow from infected mice transferred to irradiated (650 R) uninfected mice	Bone marrow from uninfected mice transferred to irradiated (650 R):	
		Infected mice	Uninfected mice (control)
5	31.0 ± 1.30*	28.0 ± 1.50	30.0 ± 2.10
14	32.0 ± 1.40	46.0 ± 2.51‡	32.0 ± 1.50
24	36.6 ± 0.82†	38.0 ± 0.82†	32.1 ± 1.43
42	31.2 ± 0.86	29.5 ± 1.12	30.4 ± 1.03

\*  $\bar{x}$  ± SD calculated from 10 mice per group per value.  
†  $P < 0.05$  compared with 24-day control.  
‡  $P < 0.001$  compared with 14-day control.

The data suggest that *T. spiralis* infection conditions the splenic microenvironment, which persists for 10 days and longer. It also suggests that infection influences the rate of stem cell colony formation as well as the number of stem cells. The transfer of bone marrow cells from infected mice into unconditioned irradiated uninfected mice resulted in delayed increase of CFU-S, suggesting that the events leading to stimulation of stem cell proliferation occur in the spleen (14 DPI) and then later in the bone marrow (24 DPI). This indicates that the bone marrow transfers performed on 24 DPI contained more stimulated bone marrow cells than transfers performed on 14 DPI. A considerable influence of malarial infection upon the CFU-S compartment in the bone marrow has been observed in mice (Silverman et al., 1987), indicating that the parasites can modulate stem cell response.

To our knowledge this is the first report that correlates CFU-S formation in the spleen with worm expulsion. Further studies are being conducted in order to identify differences in the microenvironment of spleens in uninfected and infected animals. It may be that the observed effect is functionally related to worm expulsion. Others have suggested that myeloid cell responses were associated with *T. spiralis* expulsion (Wakelin and Wilson, 1977; Wakelin and Donachie, 1981).

TABLE II. Adult worm counts in the intestines of BALB/c mice after infection with 300 *L.* of *Trichinella spiralis*.

Days postinfection			
5	14	24	42
122 ± 8.0*	20 ± 1.2	3 ± 0.8	0

\*  $\bar{x}$  ± SD calculated from 10 mice per group per value.

It appears that myeloid cells are major components in nodule formation as measured in our studies (Till and McCulloch, 1961; Bender et al., 1987). It is possible that several factors in the microenvironment may affect stem cell proliferation, especially colony-stimulating factor(s) (Bender et al., 1987; Metcalf, 1987) that bind to specific high-affinity receptors on the cell surfaces.

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## Glutathione S-Transferases in *Fasciola hepatica*

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**ABSTRACT:** Glutathione S-transferases (GST's) are widespread in the tissues of the liver fluke, *Fasciola hepatica*, and consist of multiple isozymes. Following purification to apparent homogeneity by affinity chromatography on glutathione agarose, fluke GST's were shown to comprise 2 components with molecular weights of about 25,000. Fluke GST's were immunogenic to rats, but when used as a vaccine conferred no protection on the animals against a challenge infection with *F. hepatica* metacercariae.

Glutathione S-transferases (GST's; E.C.2.5.1.18) are a group of isozymes that can detoxify a range of xenobiotics (Mannervik, 1985). They have been widely studied in mammalian tissues but have also been detected in a number of helminth parasites (Smith et al., 1986, 1987). cDNA coding for GST of *Schistosoma japonicum* has been cloned in *E. coli* (Smith et al., 1987). Interestingly, this gene shows about 42% similarity with rat GST. Moreover, the expressed cDNA product ( $M_r = 26,000$  expressed as a  $\beta$ -galactosidase fusion protein) confers partial protection (30% in terms of reduced adult worm recovery) on mice against *S. japonicum* infection when used as a vaccine in association with Freund's complete adjuvant (FCA). Thus, it seems likely that *S. japonicum* GST is a target of host protective immune responses.

We were interested in whether some of these observations on *S. japonicum* GST could be extended to another trematode, *Fasciola hepatica*. The questions we asked were: (1) was GST present in *F. hepatica* and where was it localised; (2) if present, was it secreted by the fluke; and (3) could it be used to vaccinate rats against infection? In this context, rats develop marked resistance to reinfection and they have been vaccinated by administering antigens excreted and/or secreted by the fluke (Howell, 1979; Rajasekariah et al., 1979).

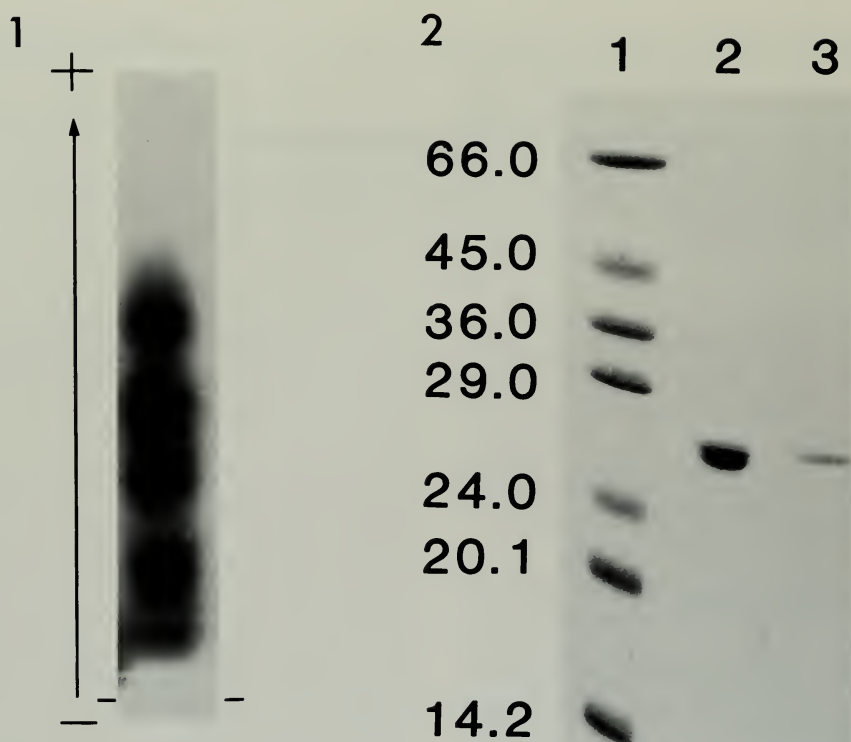
To determine if *Fasciola hepatica* contained GST, adult worms obtained from sheep were homogenized in 2 volumes of 150 mM NaCl, 50 mM Tris/HCl, pH 7.5, and the cell debris removed by centrifugation for 5 min in a micro-

fuge. GST activity in the supernatant was determined spectrophotometrically at 340 nm with reduced glutathione and 1-chloro-2,4-dinitrobenzene as substrates (Habig et al., 1974). Considerable GST activity was detected. A value of  $4.4 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$  was obtained and this is approximately 4 times the level of GST activity found in human liver (Board, 1981). In order to determine if the GST activity in adult worms was derived from multiple isozymes, the worm extract was subjected to agarose/starch gel electrophoresis (Smith et al., 1986) and specifically stained for GST activity (Board, 1980). The results of this experiment are shown in Figure 1. At least 5 zones of activity were observed migrating towards the anode. This indicates that there are multiple GST isozymes in *F. hepatica* with neutral and acidic isoelectric points. Other studies showed that the major GST isozymes from sheep liver did not comigrate with the *F. hepatica* isozymes.

The GST isozymes from *F. hepatica* were purified to apparent homogeneity by affinity chromatography on glutathione agarose (Simons and Vander Jagt, 1977). The subunit molecular size was assessed by SDS polyacrylamide gel electrophoresis (Laemmli, 1970). As shown in Figure 2 the GST's purified from *F. hepatica* contained at least 2 components equivalent to and slightly smaller than recombinant human GST 2 that has been shown from sequence analysis to have a molecular weight of 25,425 (Board and Pierce, 1987; Board and Webb, 1987).

Purified *F. hepatica* GST's were used in conjunction with FCA to vaccinate 5-wk-old male Wistar rats. Group 1 (6 rats) received the equivalent of 0.75 ml GST's (250  $\mu\text{g}$ ) in 50 mM Tris/HCl, pH 9.6, 5 mM GSH emulsified with an equal volume of FCA divided between each thigh (0.25 ml) and intraperitoneally (1.0 ml); Group 2 (5 rats) received similar injections but without GST's; Group 3 (5 rats) was left untreated. The animals were bled at weekly intervals and boosted with a similar regimen 3 wk after the first





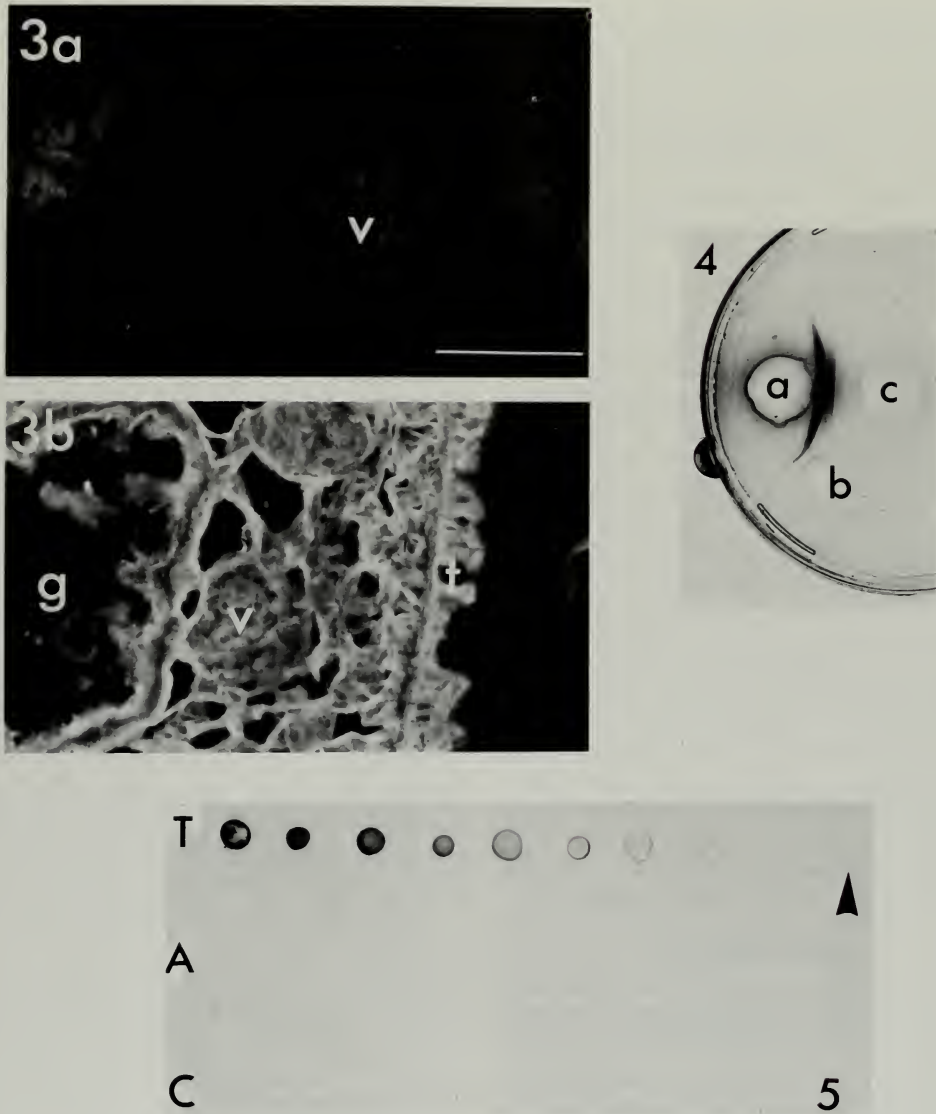
FIGURES 1, 2. 1. The glutathione S-transferase isozymes of *F. hepatica*. An extract of adult worms was subjected to electrophoresis at pH 7.4 in agarose/starch gels and enzyme activity detected *in situ*. 2. SDS polyacrylamide gel (12.5%) electrophoresis of purified GST isozymes from *F. hepatica*. Lane 1,  $M_r$  standards in kDa. Lane 2, purified GST isozymes from *F. hepatica*. Lane 3, purified recombinant human GST 2 type 1 expressed in *Escherichia coli*.

injection. One week later, when the antibody titre to GST's in the vaccinated group had reached about 1:5,000 (see below and Fig. 5), rats in each group were challenged with 25 viable metacercariae of *F. hepatica*. At necropsy 12 wk later, there was no difference in worm recovery between the 3 groups: Group 1 had  $5.75 \pm 2.1$  worms, Group 2:  $4.6 \pm 1.8$ , Group 3:  $5.5 \pm 1.4$ . Thus, despite a high titer of antibody to GST's, the rats in Group 1 exhibited no detectable resistance to infection with the parasite.

The localisation of GST's in flukes was assessed by a fluorescent antibody test using FITC-labelled sheep anti-rat IgG (Wellcome) diluted 1:20 in phosphate-buffered saline (PBS) as second antibody. Frozen sections ( $10 \mu\text{m}$ ) of worms from rats were postfixed in absolute acetone for 10 min, exposed for 1 hr to 1:15 dilutions of serum from either vaccinated rats that had been injected with GST's (see above) or controls, washed in PBS, then exposed to second antibody, washed again, and examined under a Leitz flu-

orescence microscope. The results are shown in Figure 3. In the control section there is some weak fluorescence in the vitelline follicles but elsewhere the tissues are negative. The test section indicates GST's are widespread in the tissues of the fluke including the tegument, parenchyma, and the brush border and basement membrane of the gut epithelium.

An attempt was made to determine if GST's were secreted by adult flukes. It is known that a number of antigens are secreted by the parasites and these can be identified by Ouchterlony double diffusion against infected rat serum (Howell and Sandeman, 1979). However, none of these precipitated with serum from vaccinated rats (Fig. 4). Thus, fluke GST's, if secreted by the parasite, apparently do not induce precipitins in rats. Dot blot tests of serum prepared from animals 12 wk after infection with metacercariae of *F. hepatica* failed to demonstrate the presence of antibody to fluke GST's. This tends to corroborate the view that GST's are not secreted by the parasite.



FIGURES 3-5. 3. Fluorescent antibody test for the localisation of GST's in frozen sections of *F. hepatica*. (a) Control, treated with normal rat serum. (b) Test, treated with serum from rats vaccinated with GST's. Note: GST's are widespread in the tissues (g = gut; t = tegument); some nonspecific activity is confined to the vitelline follicles (v). Scale bar = 10 μm applies to (a) and (b). 4. Ouchterlony test of serum from *F. hepatica*-infected rats (a) and rats vaccinated with GST's (b) against excretory-secretory antigens of *F. hepatica* (c). 5. Dot blot test of 2-fold serially diluted serum samples from rats vaccinated with *F. hepatica* GST's in Freund's complete adjuvant (T), rats given adjuvant only (A), and untreated rats (C) tested against 2-4-μg spots of purified *F. hepatica* GST's as antigen. The initial serum dilution was 1:10 and the endpoint 1:5,120 (arrowed).

(In other work we have found low levels of antibody to fluke GST's in naturally infected sheep.)

Antibody levels to GST's in vaccinated rats were assessed by a dot blot assay. Between 2- and 4-μg aliquots of purified *F. hepatica* GST's were spotted out on nitrocellulose using a 96-

well haemagglutination tray as a template. After drying, the nitrocellulose was blocked in 5% instant skim milk powder in Tris-buffered saline (TBS) for 30 min, then exposed to serial 2-fold dilutions of serum starting at 1:10 either for 1 hr at room temperature or 4 C overnight. Following



further washings in TBS, the nitrocellulose was incubated in a 1:300 dilution of sheep anti-rat IgG coupled to horseradish peroxidase (Silenus Laboratories, Melbourne) for 1 hr, then washed and developed using 4-chloro-1-naphthol as substrate. As can be seen in Figure 5, the vaccinated group responded strongly to the injection of purified fluke GST's, but the adjuvant only and control groups were negative.

Thus, in summary, adult *F. hepatica* contain multiple GST isozymes that are widespread in the tissues of the fluke. Unlike GST of *S. japonicum* in mice, the liver fluke enzymes do not confer any protection on rats against a challenge infection, and do not appear to be secreted by the parasite. Thus, *F. hepatica* GST's are almost certainly not host-protective antigens in rats. However, the high concentration of GST's in the fluke suggests these enzymes play an important role in the parasite's metabolism. It has been suggested (Smith et al., 1986) that *S. japonicum* GST may be involved in preventing haematin from forming large crystals that could block the parasite's gut. Because *F. hepatica* is also a blood feeder, its GST's may play a similar role. Although fluke GST's seem to be out of reach of the host immune system, they may, nevertheless, be well placed as targets for anthelmintic compounds.

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## Efficacy of a Pentaiodide Resin Disinfectant on *Cryptosporidium parvum* (Apicomplexa: Cryptosporidiidae) Oocysts *In Vitro*

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**ABSTRACT:** The resin-I<sub>5</sub> column developed at Kansas State University was tested for efficacy against oocysts of *Cryptosporidium parvum* (Apicomplexa: Cryptosporidiidae). Cesium chloride gradient-purified oocysts were passed through 1.0-cm-diameter columns with lengths of 2.5, 5.0, and 10.0 cm at 23 C. Following column passage, oocyst viability was determined both *in vitro* by excystation and *in vivo* by the ability to establish infections in suckling mice. Oocysts were found to be retained by the pentaiodide resin in a linear fashion, probably by electrostatic interactions. Linear regression analysis revealed 100% of the oocysts should be removed in such a manner using a column length of  $\geq 25.7$  cm. When compared to untreated control oocysts, <12% of the oocysts that passed through the columns appeared to be affected by the resin, as assessed by excystation. Inoculation of suckling mice with these column-treated oocysts supported the excystation data and revealed the coccidian to be viable. These results indicate that oocysts of *C. parvum* are retained on the pentaiodide column in a 1-hit manner and that, although killing of parasites may occur within the column, the greatest effect that the column may have on the parasite is as an electrostatic retention device.

The triiodide/pentaiodide resins are demand-type disinfectants that are highly effective against both viruses and bacteria (Taylor et al., 1970; Fina and Lambert, 1975; Fina et al., 1982). Marchin et al. (1983) revealed the resins also to be effective against *Giardia muris* and *G. lamblia*, with the pentaiodide inactivating more cysts than the triiodide. Because oocysts of *Cryptosporidium parvum* (Apicomplexa: Cryptosporidiidae) have been shown to occur in sewage effluents and surface waters (Madore et al., 1987), we decided to examine whether the pentaiodide is capable of removing oocysts from water.

Oocysts were obtained from the feces of a naturally infected 3-wk-old calf. After a single passage through a 3-wk-old goat, the feces was strained through a graded series of sieves and oocysts stored in 2.5% (w/v) aqueous K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> at 4 C. Prior to use, oocysts were washed once in 0.5% (v/v) aqueous Nonidet P40, separated from feces by flotation in a sucrose solution (spe-

cific gravity, 1.30), and purified further by CsCl gradients (Kilani and Sekla, 1987). All oocysts used were 6 mo old at the time of the study.

Pentaiodide was prepared by the method of Lambert et al. (1980). It can also be purchased under the trade number Pentacide from Water Technologies Corp., Ann Arbor, Michigan. The resin (9.3 meq) was poured as an aqueous slurry into glass columns (inside diameter 1.0 cm) with a small amount of fiberglass on the bottoms for the plug. Bed heights of 2.5, 5.0, and 10.0 cm were prepared. Void volumes were determined by applying aqueous solutions of dextran blue 2000 (Pharmacia, Inc.) to the columns and collecting fractions.

Oocysts purified on CsCl gradients were washed 3 $\times$  by centrifugation in distilled water and resuspended to  $4.38 \times 10^5$  oocysts/ml. Some of the oocyst suspension was used as nontreated, positive controls, whereas 40-ml aliquots of the suspensions were added carefully to each column. After collecting and discarding the void volumes, each suspension was collected in 4 10-ml aliquots after column passage. The flow rate of the columns ranges between 60 and 80 ml/min as per Marchin et al. (1983). At times 0, 10, 20, and 30 min after the suspension was passed through the columns, 1 ml of 1.0% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> was added to each of the tubes to stop iodine activity. Sodium thiosulfate was also added to one of the control tubes containing untreated oocysts. In order to determine the numbers of oocysts that passed through the columns, aliquots of 200  $\mu$ l from each of the tubes (including controls) were removed, placed in separate tubes, and an equal amount of 20% (v/v) aqueous formalin added as a preservative until oocysts could be counted by hemacytometer. All tubes were counted 10–20 $\times$ , including both positive controls with or without sodium thiosulfate treatment and oocysts in each aliquot that had passed through the columns. Data are expressed in percent of oocysts re-



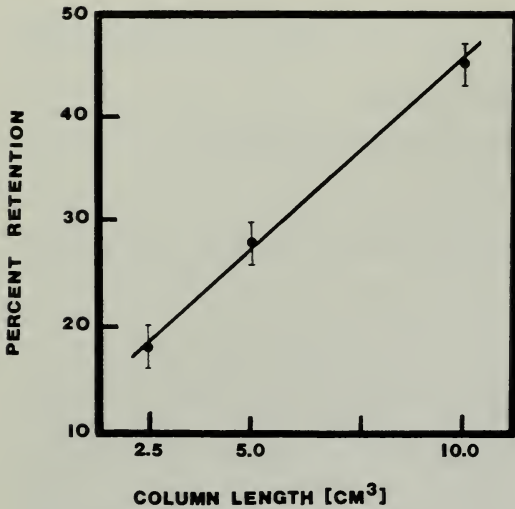


FIGURE 1. Percent retention of oocysts of *Cryptosporidium parvum* during passage through pentaioide columns of 3 different lengths. Each data point represents 40–60 hemacytometer counts and is expressed as the mean percent of oocysts retained on the column  $\pm$  the percent of the SEM.

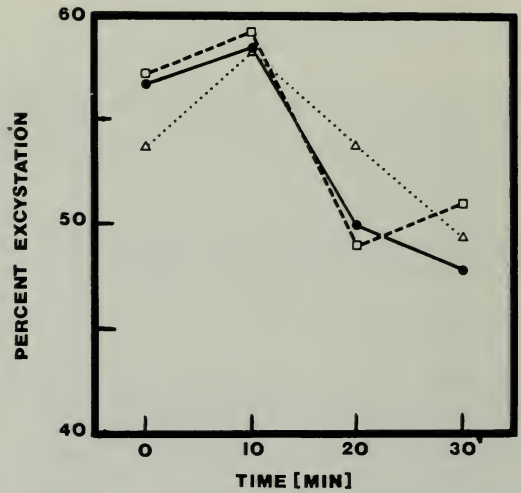


FIGURE 2. Percent of *Cryptosporidium parvum* oocysts that excysted within 60 min following passage through 3 pentaioide columns of various lengths and exposure to free iodide for 0, 10, 20, or 30 min. ●, Column length 2.5 cm; □, column length 5.0 cm; △, column length 10.0 cm.

covered from the fractions following column passage versus column length. Significance was assessed using the Mann-Whitney *U*-test, and linear regression analysis was performed on the data.

Oocysts from each of the tubes (see above) were concentrated by centrifugation and resuspended in 1.0 ml phosphate-buffered saline (PBS). An equal amount of 0.5% (w/v) trypsin–1.5% (w/v) sodium taurocholate was added to each tube, and the tubes were then incubated for 60 min at 37 C. Following excystation, the volume in each tube was adjusted to 5.0 ml with formalin to inactivate trypsin and preserve oocysts until counted. Oocyst suspensions from each of the tubes were examined by microscopy and the percent of unexcysted (intact) versus excysted (empty or excysting) oocysts determined by microscopy for the first 500 oocysts encountered from each tube.

In order to test oocyst viability following column passage, 5-day-old BALB/c suckling mice were used for experimental inoculations. As positive controls, 4 suckling mice were each inoculated orally by micropipette with  $1.0 \times 10^4$  oocysts in 10  $\mu$ l PBS that had been purified on CsCl gradients but not exposed to pentaioide treatment. Housed in a separate cage, 4 additional mice received the same number of oocysts that had been passed through the longest column (10 cm) and exposed for 30 min to the free iodine.

Three additional mice served as uninoculated, sentinel controls and were housed in the cage with mice that had received treated oocysts. All suckling mice were maintained with their dams in preautoclaved plastic cages with wire lids. Sterile wood shavings were used for bedding and all animals were kept on a 12-hr light/dark cycle. Mice were killed 4 days PI and squash preparations of the ilea were examined for the presence or absence of parasite developmental stages.

Figure 1 shows that removal of oocysts occurred in a linear fashion, proportional to column length. Linear regression revealed a column  $\geq 25.7$  cm long would be needed to remove 100% of the oocysts, providing the slope remains constant. These results suggest that oocysts are retained by a 1-hit phenomenon, which is probably due to electrostatic interactions. This would be consistent with data by Taylor et al. (1970) and Gerba et al. (1984) who have shown that both bacteria and viruses are retained on similar columns by this mechanism. It needs to be pointed out, however, that exposure of oocysts to 2.5%  $K_2Cr_2O_7$ , sucrose solution containing small amounts of phenol and CsCl may have modified the surface charge of the oocysts so that they may or may not be similar to oocysts found naturally in surface waters.

Figure 2 represents excystation data for the various fractions. Controls with or without so-

dium thiosulfate had excystation rates of 58.2% and 59.6%, respectively, and show that the reducing agent has little or no effect on excystation and adequate excystation still occurred despite the purification procedures. In each case, column length made no difference in percent excystation and all columns had <12% reduction, even after a 30-min wait prior to thiosulfate addition. These unexcysted oocysts may have been those that struck a bead in the column but then came loose. Iodine, probably in the form of  $I_2$ , is thought to be imparted directly to organic matter from a bead within the column and may have eventually penetrated the oocyst wall causing inviability. Although these data would be consistent with the kinetic studies on viral absorption by quaternary ammonium resins of Gerba et al. (1984), the slight reduction in excystation we noted using only 3 replicates is inherently too variable to prove this hypothesis. Our data do suggest, however, that free iodine alone is probably not responsible for reduced excystation because the effect, if real, occurred between 10 and 20 min following column passage (Fig. 2). Further reduction in excystation should have been noted at 30 min if free iodine had been solely responsible for the lack of excystation; however, no reduction was seen for 2 of the 3 column lengths.

All animals inoculated with oocysts, either untreated or passed through the pentaiodide column, became infected. None of the sentinel controls had parasite stages within the ileum. These data show that at least some oocysts passing through the column are capable of establishing infections.

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## Oxygen Uptake in Mice Infected with *Trichinella spiralis*

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**ABSTRACT:** Oxygen consumption, rectal temperature, and the level of activity in mice infected with *Trichinella spiralis* were significantly reduced below that seen in uninfected controls for periods of time during the first 30 days following infection. The differences in oxygen consumption between controls and infected animals were evident throughout the 24-hr period com-

prising day 7 postinfection. Both oxygen consumption and rectal temperature increased with decreasing level of infection. These changes in oxygen consumption, rectal temperature, and activity are discussed in terms of pathophysiologic and immunopathologic changes known to occur during the course of infection.



The intestinal phase of trichinosis is accompanied by anorexia and weight loss (Castro and Olson, 1967), intestinal malabsorption and maldigestion (Castro, et al., 1967; Castro and Gentner, 1972; Dembinski et al., 1979; and others), and enteritis (Russell and Castro, 1979). Later during infection, a severe myositis (Stewart et al., 1982) accompanies a decline in oxygen uptake by host muscle and changes in the size, number, internal structure, and levels of oxidative phosphorylation in mitochondria isolated from trichinous muscle (see Stewart, 1983). These parasite-induced alterations in host structure and function are accompanied by changes in the levels of host activity (Rau, 1983; Rau and Putter, 1984; Zohar and Rau, 1986) that should be reflected in a decline in oxygen uptake in trichinous animals. The present study was undertaken to determine oxygen consumption, body temperature, and level of activity in the *Trichinella*-infected host and their relationship to pathophysiologic and immunopathologic changes associated with trichinosis.

Oxygen consumption, body temperature, and level of activity were measured during the intestinal and muscle phase of infection in 6–8-wk-old, male ICR Swiss white mice (Harlan Sprague-Dawley, Houston, Texas) given 1,000 infective *L*<sub>1</sub> larvae of *Trichinella spiralis* per os. In addition, the first 2 parameters were measured in animals infected with 500 or 100 *T. spiralis* infective *L*<sub>1</sub> larvae. Methods for isolation of muscle larvae and infection of the host were those used previously (Kilgore et al., 1986). All animals were maintained on a 12-hr light–12-hr dark photoperiod and were provided food and water *ad libitum*, except for a 12-hr fast prior to determination of oxygen consumption. Mice were maintained individually for 2 hr prior to measurement of oxygen uptake in a 1,000-ml, darkened Erlenmeyer flask sealed with a 2-hole rubber stopper, provided with a continuous flow of humidified air. Oxygen consumption was measured as the product of the gas flow rate times the fractional difference in oxygen between the inflowing and outflowing air using a paramagnetic oxygen analyzer (Beckman, model 77S). Oxygen consumption ( $\mu$ l of oxygen consumed/min/g mouse body wt.), unless otherwise indicated, was measured between 1500 hours and 1800 hours. Following determination of oxygen consumption, ambient temperature, barometric pressure, rectal temperature of mice (Sensortek thermometer fitted with a stainless steel, ball-tip

microprobe, Clifton, New Jersey), and body weight of each mouse were recorded. The activity levels of infected and uninfected mice were assessed by placing an animal in its home cage into the center of a small animal activity monitor (Digiscan Optical Animal Activity Monitor, Omnitech Electronics, Columbus, Ohio), and the mean number of horizontal and vertical movements were recorded during 24-hr periods. Oxygen consumption, rectal temperature, and activity in infected mice were assessed on the days postinfection (PI) indicated in Table I. These parameters were measured in control animals on days 7, 12, and 14 of the experiment. In addition, oxygen consumption in infected and uninfected animals was measured at 6-hr intervals during the 24-hr period comprising day 7 PI. The significance of differences between means was statistically analyzed using the Student's *t*-test. Differences between means were considered significant at  $P \leq 0.05$ .

Oxygen consumption in mice infected with 1,000 *T. spiralis* was significantly reduced below that for uninfected animals after day 5 PI (days 7–30 PI; Table I). Mean rectal temperature in infected mice was significantly less than controls between days 7 and 14 PI (Table I). Both these parameters increased with decreasing level of infection (Table I). Oxygen consumption in mice infected with 1,000 *T. spiralis* was significantly less than controls at 6-hr intervals during the 24-hr period comprising day 7 PI (Table II). Comparison of the levels of activity, in terms of mean number of vertical and horizontal movements of mice during a 24-hr period, between controls and animals infected with 1,000 worms confirms the findings of Rau (1983). By day 11 PI the activity of infected animals had declined by close to 90% with regard to vertical movements and by almost 80% in the number of horizontal movements (Table I). Although not examined in the present study, Rau (1983) demonstrated a lower level of activity in animals infected with 400 *T. spiralis* than in those infected with 200 or 100 worms.

In the ICR Swiss white mouse, enteritis has subsided and adult worms have left the host intestine by day 18 PI (Stewart, unpubl.). In mice infected with 1,000 worms both rectal temperature and oxygen consumption on day 22 PI are significantly greater than that seen on day 14 PI. Differences in oxygen consumption seen between infected and uninfected animals were consistent throughout the 24-hr period comprising day 7

TABLE I. Mean  $\mu\text{l}$  oxygen absorbed/min/g mouse body weight, mean rectal temperature, and mean number of vertical and horizontal movements per mouse per 24-hr period in uninfected mice and in mice infected with 1,000 *Trichinella spiralis*; mean  $\mu\text{l}$  oxygen absorbed/min/g mouse body weight and mean rectal temperature in mice infected with 500 or 100 *T. spiralis*.\*

Day postinfection	Mean $\mu\text{l}$ O <sub>2</sub> absorbed/min/g body wt. (SE:n)	Mean rectal temperature (C) (SE:n)	Mean vertical movements/ 24 hr (SE:n)	Mean horizontal movements/ 24 hr (SE:n)
Controls	9.72 (0.76:17)	37.64 (0.09:24)	7,159 (847:16)	43,826 (2,825:16)
1,000-worm infection				
5	8.21 (1.04:5)	37.80 (0.19:4)	5,220 (328:4)†	37,577 (6,244:4)
7	2.80 (0.37:4)†	35.68 (0.29:16)†	2,943 (972:4)†	24,705 (748:4)†
9	2.71 (0.85:4)†	35.75 (0.53:4)†	2,589 (498:4)†	20,477 (893:4)†
11	3.60 (0.49:5)†	36.23 (0.05:3)†	832 (95:4)†	9,094 (820:4)†
12	2.39 (0.83:3)†	35.71 (0.23:3)†	2,547 (211:4)†	16,617 (1,393:4)†
14	2.05 (0.55:3)†‡	35.38 (0.22:4)†‡	908 (111:4)†	16,595 (1,673:4)†
22	4.19 (0.27:4)†‡	37.18 (0.43:4)†	283 (16:4)†	8,704 (1,116:4)†
30	5.89 (0.97:4)†	37.30 (0.09:4)	577 (212:4)†	13,476 (1,746:4)†
500-worm infection				
7	2.87 (0.30:4)†	36.30 (0.15:4)†		
9	3.91 (0.53:4)†	36.26 (0.03:4)†		
14	4.46 (0.17:4)†‡	36.03 (0.16:4)†‡		
22	5.39 (0.48:4)†‡	37.08 (0.10:4)†‡		
100-worm infection				
7	4.36 (0.64:4)†	36.07 (0.09:4)†		
9	4.66 (0.95:4)†	36.23 (0.03:4)†		
14	5.42 (0.38:4)†	36.63 (0.13:4)†‡		
22	6.13 (0.62:4)†	37.90 (0.25:4)†‡		

\* Standard errors (SE) and number of samples per mean (n) are shown.

† Differs significantly from corresponding value for controls,  $P \leq 0.05$ .

‡ Differs significantly from each other, within columns and within infection groups.

PI, indicating that such differences were not due to a shift in the daily oxygen consumption cycle in infected animals (Table II). This same relationship is evident in mice infected with 500 or 100 worms (Table I). Pathophysiologic (see Castro and Bullick, 1983) and immunopathologic (see Wakelin and Denham, 1983) changes in the host bowel accompanying the intestinal phase of infection may underlie these initial dramatic declines in oxygen consumption and body temperature.

Anorexia (Castro and Olson, 1967) and intestinal malabsorption (Castro et al., 1967) decrease the nutrients available to the host during the enteric phase of trichinosis. This decline in available energy may underlie a decrease in the level

of host metabolism, which is reflected by a drop in oxygen consumption and body temperature in the host. A portion of the smaller pool of energy available in the infected host is used to support physiologic and immunologic responses to parasite-induced alterations in the bowel, further lowering energy available for normal physiologic processes such as muscular activity. Thus, a sharp decline in host activity is observed during the intestinal phase of trichinosis.

After day 22 PI, body temperature in infected animals (regardless of level of infection) has risen to or near that seen in control animals. With the subsidence of parasite-induced alterations in the intestine, the level of oxygen consumption on day 22 PI may reflect primarily the decreased

TABLE II. Mean  $\mu\text{l}$  oxygen absorbed/min/g mouse body weight at 6-hr intervals during the 24-hr period comprising day 7 PI in uninfected mice and in mice infected with 1,000 *T. spiralis*.\*

Group	$\mu\text{l}$ O <sub>2</sub> adsorbed/min/g body wt.			
1,000-worm infection	2400 hours	0600 hours	1200 hours	1800 hours
	3.47 (0.32:3)†	2.96 (0.91:4)†	3.12 (0.38:3)†	3.49 (0.54:3)†
Controls	5.86 (1.26:4)	5.81 (1.26:4)	5.39 (0.41:4)	8.31 (0.58:3)

\* Standard errors (SE) and number of samples per mean (n) are shown.

† Differs significantly from corresponding value for controls,  $P \leq 0.05$ .



demands placed on host respiration by the decline in animal activity shown to be associated with the presence of muscle larvae (Zohar and Rau, 1986). This study has identified another facet of the wide-ranging pathophysiologic alterations that accompany infection of the mouse with *Trichinella spiralis*.

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## Intramuscular *Sarcocystis* sp. in Two Cats and a Dog

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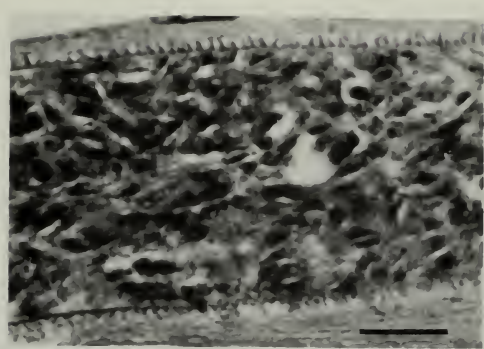
**ABSTRACT:** *Sarcocystis* sp. was diagnosed in the skeletal muscle of a cat and myocardium of a dog and cat. The cysts were similar in size and structure when examined by light and transmission electron microscopy. The 3 animals were debilitated and probably immunocompromised due to pancytopenia or terminal neoplasia.

Apicomplexan parasites of the genus *Sarcocystis* produce intramuscular cysts in mammals,

reptiles, birds, and fish (Dubey, 1976), but the intramuscular phase of the parasite cycle is rare in dogs and cats (Kirkpatrick et al., 1986). Recently, we observed the unusual presence of sarcocysts in the skeletal muscle of a cat and myocardium of a dog and cat.

A 5-yr-old castrated male cat was admitted to the University of Georgia Small Animal Teaching Hospital because of rear leg lameness. A neo-

FIGURES 1, 2. Photomicrographs of *Sarcocystis* sp. cysts. 1. Light microscopic photomicrograph of a cyst of *Sarcocystis* sp. in striated muscle from a cat with malignancy of the femur. Note striations in the cyst wall. H&E; bar = 10  $\mu$ m. 2. Transmission electron photomicrograph of a cyst of *Sarcocystis* sp. in a skeletal myofibril (SM) in the cat. Observe the striated (ST) cyst wall and the ground substance (GS) that continues into the septa (arrows,  $\rightarrow$ ) within the cyst. Nuclei (N) and polysaccharide (PG) granules are identified in bradyzoites. Bar = 1  $\mu$ m.





plasm of the femur and thigh muscles was confirmed from a biopsy sample, and the leg was amputated. Histopathologic evaluation revealed lymphosarcoma involving the bone marrow and cancellous bone of the femur and the muscles surrounding the femur. Sarcocysts, 5 of which measured an average of  $280 \times 53 \mu\text{m}$  and contained large numbers of bradyzoites ( $8\text{--}10 \mu\text{m}$  long), were observed in the thigh muscles. The striated cyst wall and an associated wide homogenous eosinophilic ground substance measured  $1.5\text{--}2.0 \mu\text{m}$  thick as determined from hematoxylin and eosin stain (H&E). The bradyzoites in the cysts stained with periodic acid-Schiff (PAS), but the cyst wall did not. Striations in the cyst wall were accentuated with mucicarmine stain. The bradyzoites were separated by homogenous eosinophilic spaces that were interpreted by brightfield light (Fig. 1) and transmission electron microscopy (Fig. 2) to be septa. No sarcocysts were evident in the heart when the cat was examined at necropsy several days later.

Formalin-fixed tissue from a 5-yr-old castrated male cat with a prior history of leukopenia, anemia, and thrombocytopenia was sent to the University of Georgia Diagnostic and Investigational Laboratory in Tifton, Georgia. Primary lesions found on light microscopic examination of the major organs of the cat were centrilobular necrosis of hepatocytes and focal accumulations of lymphocytes in the myocardium. Microscopic examination of the heart disclosed sarcocysts in the myocardium. The striated cyst wall enclosed numerous bradyzoites. Morphometrically, cysts were similar to those in the skeletal muscle of the cat previously described. There was no inflammatory reaction in the immediate area of the sarcocysts.

Metastatic neoplasia was suspected in a 2-yr-old spayed female Doberman pinscher, which was referred to the University of Georgia Small Animal Teaching Hospital. Pulmonary adenocarcinoma and congestive cardiomyopathy were diagnosed; the neoplasm was considered untreatable. Euthanasia was performed. Sarcocysts, 3 of which measured an average of  $191 \times 46 \mu\text{m}$ , were observed in the myocardium. The sarcocyst wall and ground substance were  $1.5\text{--}2.0 \mu\text{m}$  thick. The ground substance alone was not as wide in the dog's cysts as in the cats' cysts. The sarcocysts contained numerous bradyzoites that measured  $11\text{--}12 \mu\text{m}$  in length and were compartmentalized by eosinophilic septa. No degeneration of myo-

cardium nor inflammatory cell infiltration was associated with the cysts.

*Sarcocystis* spp. have an obligatory 2-host life cycle in which an intermediate host ingests sporocysts that undergo merogony predominately in vascular endothelium, and merozoites encyst in cardiac and skeletal muscle where they divide by endodyogeny to form metrocytes and eventually bradyzoites (Tadros and Laarman, 1982; Fayer and Dubey, 1986). Definitive hosts (carnivores or omnivores) become infected by ingesting bradyzoites contained within muscle cysts of prey animals (McKenna and Charleston, 1980; Hilali et al., 1982). We could find only 1 report of an intramuscular stage of *Sarcocystis* in dogs (Sahasrabudhe and Shah, 1966) and only rare reports in cats have been described (Kirkpatrick et al., 1986).

Most intramuscular cysts described in these cases did not elicit any inflammatory response. Sarcocysts previously reported in 2 cats were found incidental to examination of tissue for neoplasia (Kirkpatrick et al., 1986). Each of the animals described in our report was debilitated and immunocompromised, the result of severe pancytopenia in 1 cat and terminal metastatic neoplasia in the dog and other cat. Because the life cycle of *Sarcocystis* spp. usually is considered to be a predator-prey (carnivore-herbivore or omnivore) interaction or scavenger-carrion cycling, the unusual infection of the dog and cat may be due in part to debilitation or immunosuppression.

Taxonomy of *Sarcocystis* based on structure as determined by light microscopy is imprecise, but observations from transmission electron photomicrographs on the cysts, cyst walls, bradyzoites, and septa between bradyzoites suggest that the sarcocysts seen in the 2 cats reported here were structurally similar. Differences observed in the sarcocysts from the dog as compared to those of the cat were a thinner layer of ground substance associated with the cyst wall and slightly larger bradyzoites. The differences were considered within the limits of normal biologic variations. The cyst wall striations, large size of the cysts and bradyzoites, and compartmentalization of bradyzoites by septae differentiated the *Sarcocystis* from *Toxoplasma gondii* and *Hammondia* sp. The species of *Sarcocystis* infecting the cats and the dog could not be determined.

We acknowledge Dr. J. L. Clark, Cairo, Geor-

gia, for providing the tissues for microscopic examination from one of the cats.

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## Immunological Relationship of *Plasmodium inui* with Two Other Quartan Malaria Parasites, *P. malariae* and *P. brasilianum*

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**ABSTRACT:** A series of monoclonal antibodies was produced against sporozoites of the OS strain of *Plasmodium inui*, a simian quartan malaria parasite, and used to characterize the circumsporozoite protein of this parasite. The results confirm that the immunodominant epitope of the circumsporozoite protein of *P. inui* is immunologically distinct from those of 2 other quartan parasites, the human *P. malariae* and simian *P. brasilianum*, which are identical.

*Plasmodium inui* is a malaria parasite of Old World monkeys. Because of its quartan periodicity and growth in rhesus monkeys, it has been considered a suitable laboratory model of infection with the human quartan malaria parasite *P. malariae* (Schmidt et al., 1980). A number of strains of *P. inui* have been described (Coatney et al., 1971). In the present study, we used the OS strain (Collins et al., 1981).

The circumsporozoite (CS) protein is the major antigen responsible for the stage- and species-specific protective immune response induced by vaccination with sporozoites of mammalian malaria (Nussenzweig and Nussenzweig, 1986).

Recently, we found that monoclonal antibodies (moAB's) produced against sporozoites of *P. malariae* (Cochrane et al., 1984a) and *P. brasilianum* (Cochrane et al., unpubl. data), a quartan parasite of New World monkeys, did not react with sporozoites of *P. inui* (OS) by indirect im-

munofluorescence (IFA). To understand this apparent lack of identity between the CS proteins of *P. inui* and the other 2 quartan malaria species, we produced a series of moAB's against *P. inui* sporozoites. We have used these moAB's to characterize the CS protein of *P. inui* and to confirm that the immunodominant epitope of the CS protein of this parasite is immunologically distinct from those of *P. malariae* and *P. brasilianum*, which are identical (Cochrane et al., 1985; Lal et al., 1987).

MoAB's against *P. inui* sporozoites, recovered by salivary gland dissection of infected *Anopheles dirus* mosquitoes, were produced following described procedures (Kohler and Milstein, 1975). Six hybridomas secreting moAB's, all IgG<sub>1</sub>, were obtained. Ascites fluid recovered from hybridoma-bearing mice reacted by IFA with glutaraldehyde-fixed sporozoites of *P. inui* at high dilutions (1:10<sup>6</sup>). None of the 6 moAB's reacted with glutaraldehyde-fixed sporozoites of *P. malariae* or *P. brasilianum*. They also failed to react with sporozoites of rodent (*P. berghei*) and simian (*P. cynomolgi*) malaria, and the other 3 human malaria species.

The CS protein of *P. inui* was identified by Western blot analysis as described for malaria sporozoites (Cochrane et al., 1984b). All 6 of the anti-*P. inui* moAB's recognized the same 2 pro-



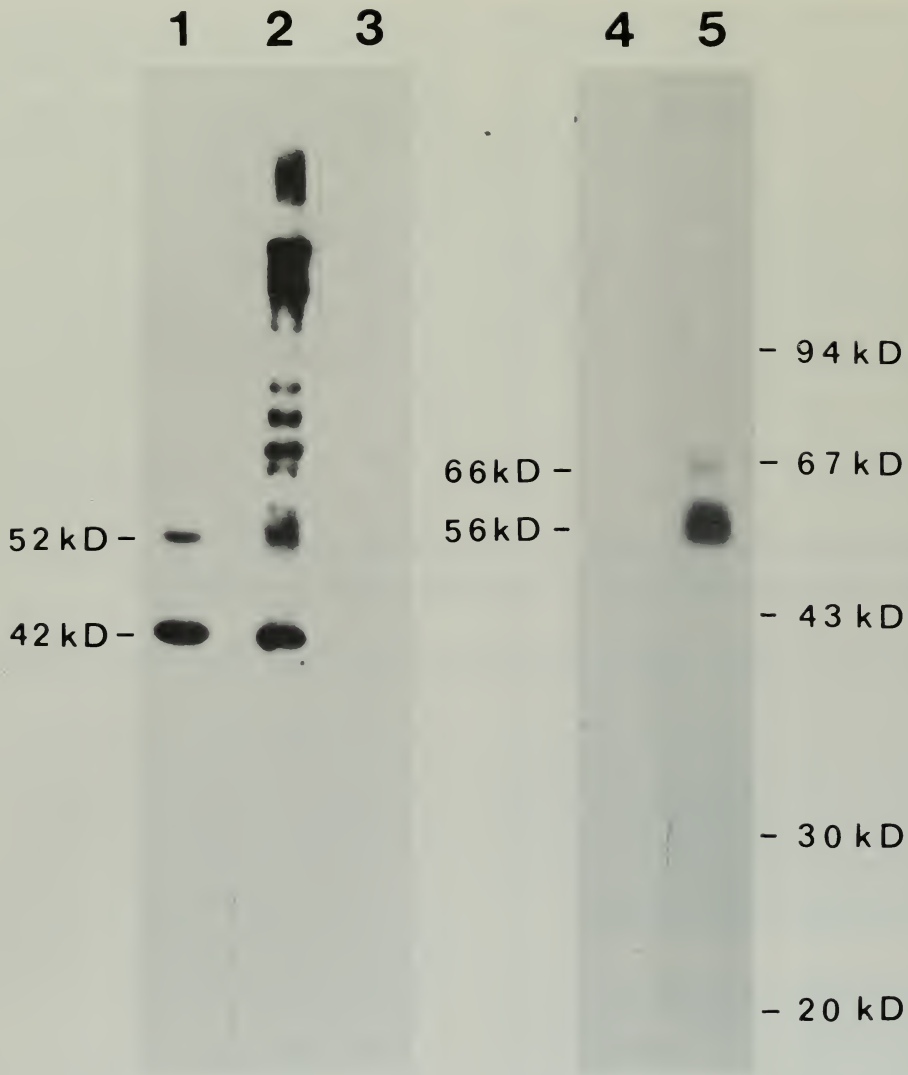


FIGURE 1. Western blot analysis of sporozoite extract of *P. inui* and *P. brasilianum* as described in text. Two proteins with molecular weights of 52 kDa and 42 kDa were identified in extracts of *P. inui* sporozoites using either the homologous moAB's (track 1) or the homologous mouse immune serum (track 2). These proteins were not identified by moAB raised against sporozoites of *P. berghei* (track 3). Two proteins with molecular weights of 66 kDa and 56 kDa were identified in extracts of sporozoites of *P. brasilianum* by the homologous moAB's (track 5) but not by the *P. inui* moAB's (track 4).

teins with molecular weights of 52 kDa and 42 kDa in extracts of *P. inui* sporozoites (Fig. 1, track 1). The same molecules were also detected by serum of mice immunized with sporozoites of *P. inui* (track 2), but not by control moAB raised against sporozoites of *P. berghei* (track 3).

None of the *P. inui* moAB's recognized the CS proteins of *P. brasilianum* in extracts of these parasites (Fig. 1, track 4) that were identified (molecular weights 66 kDa and 56 kDa) by

moAB's previously raised against sporozoites of the Colombian strain of *P. brasilianum* (track 5).

An immunodominant repetitive epitope on the CS protein of *P. inui* was demonstrated using the anti-*P. inui* moAB's in a competitive binding and a 2-site immunoradiometric assay (Zavala et al., 1983). In the competitive binding assay, incubation of each of the unlabeled moAB's with sporozoite extract bound to wells of microtitre plates totally inhibited the binding of the ra-

diolabeled homologous moAB as well as the binding of each of the other 5 radiolabeled moAB's. These results indicate that each of these moAB's recognizes the same epitope on the CS protein of *P. inui*. Results of the 2-site immunoradiometric assay indicate that the epitope, recognized by each of the moAB's, is repeated at least twice on the same CS molecule.

As shown in the present study, the CS protein of *P. inui* is analogous to those of other mammalian malaria parasites in both its apparent molecular weight and the presence of an immunodominant repetitive epitope. Perhaps more importantly, the results confirm and extend our earlier observations indicating that the CS protein of *P. inui* is immunologically distinct from those of *P. malariae* and *P. brasilianum*.

Earlier we had also found that most moAB's produced against blood stages of *P. brasilianum* reacted with blood stages of *P. malariae*, but that only a small number of these moAB's reacted with *P. inui* (OS) blood stages (Cochrane et al., unpubl. data). We have furthermore recently observed that a high percentage of moAB's produced against blood stages of *P. cynomolgi* (Berok) react with blood stages of *P. inui*, but few of these moAB's react with blood stages of *P. brasilianum* (Kamboj et al., unpubl. data). *Plasmodium inui* has been considered a biologic model for *P. malariae* because of its quartan periodicity and chronic long-term infections. However, taken together, our data indicate that *P. inui* should not be used as an immunologic model for *P. malariae* and raise the possibility of a closer evolutionary relatedness of *P. inui* to tertian rather than quartan malaria parasites.

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## Hemolytic and Coagulation Properties of *Sphaeridiotrema globulus* (Trematoda)

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**ABSTRACT:** Metacercariae of *Sphaeridiotrema globulus* (Trematoda) were obtained from naturally infected *Goniobasis virginica* (Pleuroceridae). Excysted metacercariae were placed individually on blood-agar plates. After 8 hr of incubation at 42 C the plates exhibited  $\beta$ -hemolysis. The reaction zone was approximately 1.5 mm in diameter. Laboratory-reared mallard ducks (*Anas platyrhynchos*) were infected with *S. globulus*. Mallards developed fatal sphaeridiotremiasis and demonstrated increased prothrombin time on days 3 and 6 postinfection.

Lake Musconetcong, located in Morris County, New Jersey, has been the site of mortalities of mute swans, *Cygnus olor*, and whistling swans, *Olor columbianus*. The deaths were caused by ulcerative hemorrhagic enteritis and severe anemia produced by the trematode *Sphaeridiotrema globulus* (Roscoe and Huffman, 1982, 1983).

We report the ability of excysted metacercariae of *S. globulus* to produce  $\beta$ -hemolysis on blood-agar, and the effect of the adult parasite on the proenzyme prothrombin in experimentally infected mallard ducks (*Anas platyrhynchos*).

$\beta$ -Hemolysis is caused by proteins termed hemolysins that cause the release of hemoglobin from erythrocytes. The  $\beta$ -hemolysis reaction is observed when all erythrocytes are lysed and a transparent zone appears in the agar.

Metacercarial cysts of *S. globulus* were obtained from naturally infected *Goniobasis virginica* snails (Huffman and Fried, 1983). Excystation was induced for separate groups at 42 C and 37 C using the following pretreatment sequence: individual cysts were placed 100 per petri dish (3.5 cm diameter) with (1) 3%  $\text{NaHCO}_3$  for 30 min, (2) 1% acid pepsin for 10 min, and (3) 1% acid pepsin for 30 min. The cysts were then transferred to petri dishes containing 4 ml of 0.5% bile salts plus 0.5% trypsin in Earle's BSS adjusted to pH 7.8 with 7.5%  $\text{NaCHO}_3$  (Fried and Huffman, 1982). Cysts were examined within 1 hr. The highest excystation rate was found using the 3%  $\text{NaHCO}_3$  for 30 min at 42 C. From 5 to

10 excysted metacercariae were transferred through sterile Locke's solution containing penicillin (200 units/ml) and streptomycin (200  $\mu\text{g}/\text{ml}$ ) and then placed individually on 8.5-cm-diameter blood-agar plates containing a suspension of sheep red blood cells (Mayer and Myle Laboratories, Coopersburg, Pennsylvania). As controls, the blood-agar was also spotted with the excystation medium and the Locke's solution containing the antibiotics. These plates were incubated at 42 C and checked at 2, 8, and 24 hr. All areas on which the parasites were placed exhibited  $\beta$ -hemolysis after 8 hr of incubation with a reaction zone of approximately 1.5 mm. The controls showed no reaction.

To ascertain the effect of adult *S. globulus* on the clotting factor prothrombin, the thromboplastin test (Sigma Co., St. Louis, Missouri) was

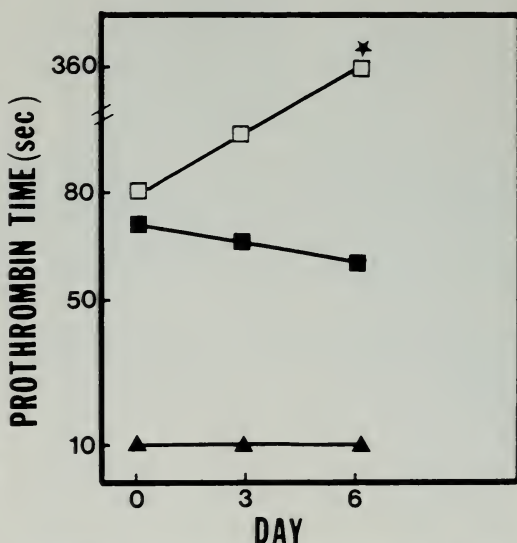


FIGURE 1. Mean prothrombin time (sec) in serum from mallards infected with *S. globulus* metacercariae (□, 2,500), control serum (■), and human serum (▲). ★, Animals died of the infection.

performed using laboratory-reared adult mallard ducks. Five mallards were each given a lethal dose of 2,500 metacercariae (Huffman and Roscoe, 1986) via esophageal catheter. Five mallards were designated as controls and received no metacercarial cysts; human serum was also used as a second control. Blood samples were taken from the brachial wing vein prior to infection and on days 3 and 6 postinfection. Mallards infected with *S. globulus* demonstrated increased prothrombin time on days 3 and 6 postinfection (Fig. 1). According to Guyton (1971), prothrombin time is the length of time required for the blood to clot, which depends on the amount of prothrombin in blood. The longer the prothrombin time, the less amount of prothrombin available. Prothrombin time increased to 6 min on day 6, the day the animals died of the infection.

A possible mechanism of hemorrhage induced by *S. globulus* in waterfowl is explained by the following process. The parasite may initially cause mechanical damage to the mucosal and submucosal tissues and rupture capillaries. Proteases of parasite origin may then act as anticoagulants to maintain intestinal hemorrhage. The results of this study indicate that  $\beta$ -hemolysis may be involved in the initial inductive stages

of the pathology of this trematode infection along with the inhibition of prothrombin.

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## Degenerative Changes in Lymphatic Endothelium of Jirds Infected with *Brugia pahangi*

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**ABSTRACT:** The quantitative changes of cytoplasmic vesicles and vacuoles in lymphatic endothelial cells of the mongolian jirds associated with *Brugia pahangi* infections were observed by transmission electron microscopy. The present study revealed a decrease in the proportion of cytoplasm occupied by vesicles and in the number of cytoplasmic vesicles in endothelial cells from lymphatic vessels harboring *B. pahangi* at 3, 4, and 10 mo after infection (3.55, 3.36, and 2.55 vesicles/ $\mu\text{m}^2$ , respectively) when compared with cells from uninfected control vessels (7.03 vesicles/ $\mu\text{m}^2$ ). On the contrary, there was an increase in the area of vacuoles in endothelial cells of jirds at 3, 4, and 10 mo postinfection. The mean  $\pm$  SD diameter of vesicles in cells from lymphatic vessels at 10 mo after infection was

significantly smaller ( $78.6 \pm 5.6$  nm) compared to vesicles in uninfected vessels ( $87.5 \pm 9.7$  nm).

Histopathological changes in lymphatic vessels in experimental animal models infected with *Brugia* nematodes have been extensively studied (Schacher and Sahyoun, 1967; Rogers and Denham, 1974; Denham and Rogers, 1975; Rogers et al., 1975; Ewert et al., 1980; Sakamoto, 1980; Vincent et al., 1980; Folse et al., 1981). However, a complete explanation of the role of lymphatic endothelial cells in filarial infection has not yet



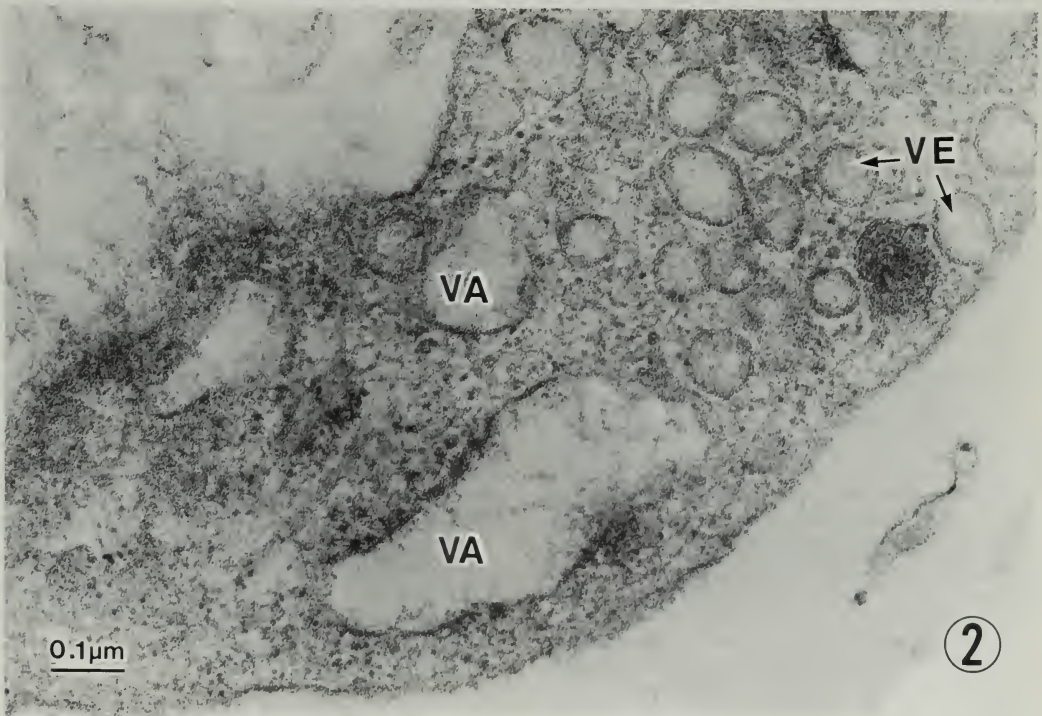
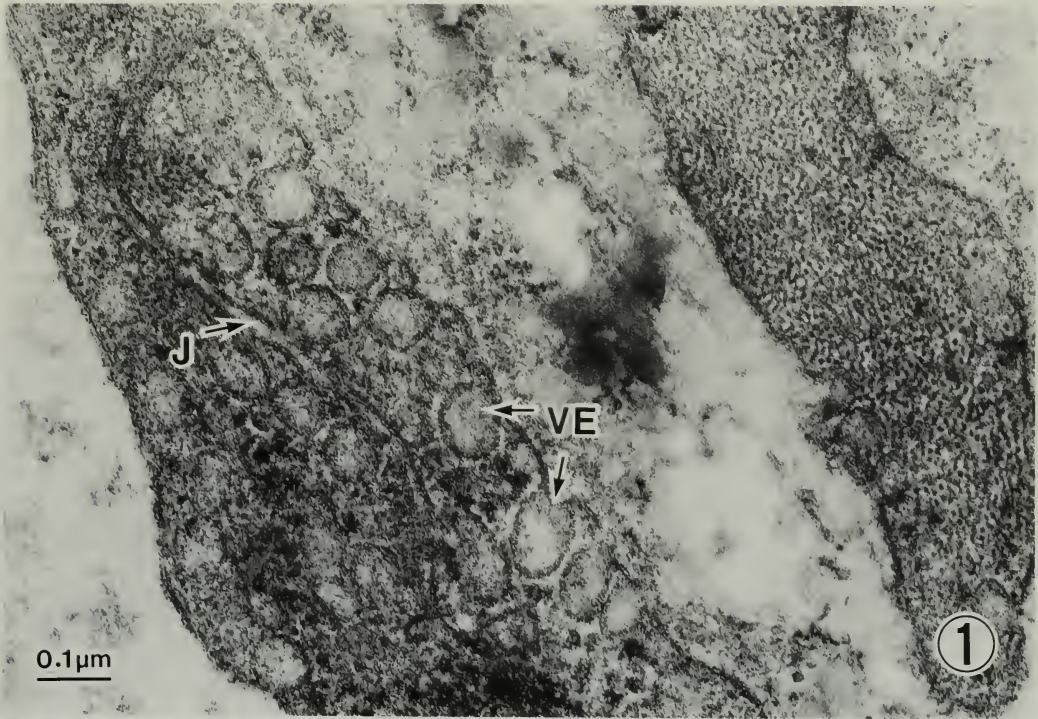
been given. Sakamoto et al. (1985) reported a decrease in the number of cytoplasmic vesicles in endothelial cells from lymphatic vessels of cats harboring *B. malayi*. Such changes in cellular morphology might hamper the efficiency of vessels that collect and transport edematous fluid in affected limbs. The present study describes quantitative changes of cytoplasmic vesicles and vacuoles in lymphatic endothelial cells at various times of infection in jirds infected with *B. pahangi*. Each of 25 1-yr-old female jirds was infected with *Brugia pahangi*. Fifty infective *B. pahangi* larvae obtained from infected *Aedes aegypti* mosquitoes were inoculated subcutaneously into the dorsum of the left foot. Five 1-yr-old, uninfected female jirds were used as a control group. Regional lymph vessels were delineated by injection of 5% patent blue dye into the interdigital space of a hind foot of anesthetized jirds. Immediately before animals were killed, lymph vessels were perfused with 2% glutaraldehyde fixative by using a fine glass tube. Five specimens of lymph vessels lying between the hind foot and the popliteal lymph node were chosen at random for electron microscopic study. Thin sections, 60–90 nm, were cut with glass knives, stained with uranyl acetate (Hayat, 1981) and lead citrate (Reynolds, 1963), and observed in a JEM 100 X transmission electron microscope (Nippon Denshi Ltd., Japan). Random photographs (15–20/specimen) were taken of the lymphatic endothelial cells and printed to a final magnification of 144,000. The total endothelial cytoplasmic area of nonnuclear regions of each micrograph was calculated by using a computer graphic system (Cosmozone, Nikon Ltd., Japan). Both the total number and the area of vesicles were measured, and the ratios per unit area were calculated. Vacuoles, which were larger than vesicles, irregular in shape, and usually containing degenerating organelles, were counted separately and subjected to the same analysis as vesicles. Statistical analysis was performed where appropriate using Wilcoxon's rank-sum test or Scheffe's test.

The mean  $\pm$  SD diameter of the vesicles in endothelial cells of control jirds was  $87.5 \pm 9.7$  nm. This did not differ significantly from the mean  $\pm$  SD diameter of  $87.2 \pm 7.9$ ,  $80.9 \pm 7.7$ ,  $82.6 \pm 8.0$ , and  $82.0 \pm 7.2$  nm seen in lymphatic endothelial cells at 1, 2, 3, and 4 mo after infection, respectively. This corresponded to that reported previously in lymphatics of rat kidneys

(80–100 nm: Albertine and O'Morchoe, 1980) and dog paw (84 nm: Yang et al., 1981). However, the mean  $\pm$  SD diameter of the vesicles in cells at 10 mo after infection was  $78.6 \pm 5.6$  nm, which was significantly decreased compared to the diameter of vesicles in endothelial cells of control jirds ( $P < 0.05$  by Scheffe's test). This difference might be the result of long-term *B. pahangi* infection. Most lymph vessels in jirds infected with *B. pahangi* at 3, 4, and 10 mo after infection were dilated and brittle. The uniform cytoplasmic vesicles of endothelial cells in control jirds can be seen in Figure 1. The mean number of vesicles per  $\mu\text{m}^2$  in control, and in months 1, 2, 3, 4, and 10 after infection were 7.03, 7.78, 6.61, 3.55, 3.36, and 2.55, respectively. There were significantly fewer vesicles at 3, 4, and 10 mo than in endothelial cells from vessels of control jirds ( $P < 0.01$ ). The percentages of vesicle area over cytoplasmic area at 1 and 2 mo were 3.85 and 3.45%. These did not differ significantly from 4.70% seen in cells of control jirds. However, the percentages of vesicle area over cytoplasmic area at 3, 4, and 10 mo were 1.76, 1.80, and 1.82%, respectively. This is a significant decrease ( $P < 0.01$ ).

It is known that cytoplasmic vesicles are responsible for the transportation of molecules across the endothelial cell walls of blood vessels. Several authors have postulated that these vesicles also played an important role in transportation of molecules across the endothelial cells of lymphatic vessels (Albertine and O'Morchoe, 1980; Yang et al., 1981). On the other hand, Casley-Smith and Window (1976) have suggested that fluid and protein escapes from the blood vessels, affected by histamine and burning, through the endothelial intercellular junctions. The openings in these junctions have been ascribed to the contraction of endothelial cells, which was also seen in lymphatic endothelium (Majno et al., 1969; Joris et al., 1972) and in mesothelium (Casley-Smith and Bolton, 1973).

By using electron microscopy to study changes in lymphatic endothelial cells in domestic cat infected with *B. malayi*, Sakamoto et al. (1985) revealed a decrease in the number of cytoplasmic vesicles and an increase in area occupied by vacuoles at 3 mo after infection. Based on these results, they implied that those alterations of cells might render them less effective in transporting edematous fluid away from filaria-affected limbs and contributed to lymph edema and collagen



FIGURES 1, 2. 1. Endothelial cells from an uninfected lymph vessel. Numerous small vesicles were seen in the cytoplasm. VE: vesicles; J: junction; L: lumen. 96,000 $\times$ . 2. Endothelial cells from the lymph vessels of a jird infected with *B. pahangi*. Large irregular vacuoles were seen. VA: vacuoles; VE: vesicles; L: lumen. 96,000 $\times$ .



accumulation that were prominent in lymphatic filariasis. To characterize further the alterations of lymphatic endothelial cells in jirds infected with *B. pahangi*, vacuoles were observed in the present study. Figure 2 shows the cytoplasmic vacuoles that could be differentiated from normal vesicles in the cytoplasm of endothelial cells of uninfected jirds. Large, irregular vacuoles were often seen in cells of infected jirds. The proportion of cytoplasm occupied by vacuoles in control jirds was 2.96%. The proportions at 1 and 2 mo after infection were 3.94 and 2.43%. These percentages were not significantly different from the control jirds when subjected to statistical analysis. However, the percentages of vacuole area over the nonnuclear cytoplasmic area at 3, 4, and 10 mo after infection were 7.96, 14.20, and 8.49%, respectively. A remarkable increase was seen in the proportion of cytoplasm occupied by vacuoles in endothelial cells of jirds at 3, 4, and 10 mo. Casley-Smith and Window (1976) reported that there was a marked increase in the number of vacuoles in endothelial cells from blood capillaries following injury by burns. In our study, there were significant increases in the area of vacuoles of cells at 3, 4, and 10 mo after infection. This corresponds to the report by Sakamoto et al. (1985) that there was a remarkable increase in the area of vacuoles in lymphatic endothelial cells of cat infected with *B. malayi* at 3 mo postinfection.

Our study indicates that the decrease in the number of vesicles and the increase in the area of vacuoles in lymphatic endothelial cells of jirds were caused by *B. pahangi* infection.

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## A Simple Technique for Recovering Larval Ascaridoid Nematodes from the Flesh of Marine Fish

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**ABSTRACT:** This paper describes and evaluates the efficiency of a simple technique for recovering larval ascaridoid nematodes (*Anisakis simplex* and *Pseudoterranova decipiens*) from the flesh of marine fish. The technique involves mechanical disintegration of the flesh in a domestic food processor, followed by visual inspection of diluted portions of the resulting homogenate under short-wave ultraviolet light. The nematodes, which remain intact, fluoresce brightly and are easily detected, particularly if the musculature has been frozen and thawed previously. The technique recovers a much higher proportion of the total number of nematodes than candling and slicing, is more rapid than pepsin-HCl digestion, and would therefore be suitable for large-scale surveys of ascaridoid nematodes in the flesh of marine fish.

Most surveys of larval ascaridoid nematodes in marine fish have relied on candling or candling and slicing techniques (Power, 1961) to recover nematodes from the host's musculature (Templeman et al., 1957; Young, 1972; Arthur et al., 1982; McClelland et al., 1983). The proportion of nematodes recovered by these techniques depends on several factors, yet in few surveys has the accuracy of the examination procedure been estimated (McClelland et al., 1983). Consequently, problems occur when investigators working on the same species compare the results of surveys; apparent differences in nematode abundance may reflect variable accuracy of the examination procedure rather than any real difference in parasite numbers. Elimination of this difficulty requires a more effective examination technique that consistently gives accurate counts of nematodes in the flesh.

Pepsin-HCl digestion (Stern et al., 1958) is more accurate than candling and slicing and probably recovers all nematodes from the flesh. This technique has been used extensively for experimental studies where accurate nematode counts are required from relatively small numbers of fish (Smith and Wootten, 1975; Arthur et al., 1982; McClelland et al., 1983). However, conducting several pepsin-HCl digestions is tedious, expensive, and therefore unsuitable for large-scale surveys that often involve examining the flesh of hundreds of fish.

This paper describes and demonstrates the efficiency of a simple technique for finding larval ascaridoid nematodes in the flesh of marine fish. The technique, which is a modification of a method described by Apold et al. (1984), recovers a much higher proportion of the total number of nematodes than candling and slicing and is more rapid than pepsin-HCl digestion.

The initial stages of the technique involve mechanical disintegration of the flesh in a domestic food processor. Briefly, skinned fillets and napes (=hypaxial musculature, or muscle surrounding the body cavity) were weighed (nearest gram) and placed in a domestic food processor (Moulinex Model 663, Cuisinart Model DCL 10+, or similar) equipped with a blunt-edged plastic dough blade. An amount of hot (50–60 °C) tap water equivalent to twice the weight of the flesh was added, and depending on the size of the fish, the muscle was thoroughly blended for 1–2 min at medium speed. Apold et al. (1984) used a sieving process to recover "*Phocanema*" (= *Pseudoterranova*) *decipiens* nematodes from the resulting homogenate; however, in the present study the small mesh sizes necessary to recover larvae of *Anisakis simplex* tended to clog and the worms were difficult to distinguish from muscle fibres. The following procedure was more efficient: the total homogenate was divided into small quantities (200 ml) and these were transferred to separate clear glass trays (35 × 25 × 6 cm) containing 200 ml of tap water. The trays were placed on a black surface. The contents of each tray were agitated vigorously with hand-held forceps and examined under high-intensity (> 500 µW/cm<sup>2</sup>), short-wave (365 nm), ultraviolet (UV) light, either in a darkened room or in a viewing chamber (see Pippy, 1970). Eye protection, appropriate to the type of UV lamp being used, should be worn during examination. The nematodes, which remained intact, fluoresced a brilliant bluish-white (Sinnhuber and Law, 1949) and were easily detected, particularly if the fillets had previously been frozen and thawed (Pippy, 1970). Fish bones and small fragments of nerve tissue also flu-



TABLE I. Numbers of larval ascaridoid nematodes found in the flesh of cod, *Gadus morhua*, by candling and slicing followed by mechanical disintegration of the flesh and reinspection under short-wave ultraviolet light ( $n = 505$ ).

Nematode species	Body component	Total worms found	Percent found by	
			Candling and slicing	Mechanical disintegration plus UV light
<i>Anisakis simplex</i>	Fillets	61	42.6	57.4
	Napes	188	78.7	21.3
	Fillets and napes combined	249	69.9	30.1
<i>Pseudoterranova decipiens</i>	Fillets	230	95.2	4.8
	Napes	15	93.3	6.7
	Fillets and napes combined	245	95.1	4.9

oresced but, with experience, they were easily distinguished from nematodes. Preliminary trials showed that the UV light does not penetrate tissues deeply, and that approximately 20% of the nematodes will not be detected if the trays contain large quantities of undiluted homogenate. Therefore, during examination the proportions of homogenate to water described above should be strictly adhered to and the depth of the material in the trays should not exceed 1 cm.

To determine what proportion of the total number of nematodes present in the flesh are found using the new technique, several blind trials were conducted with larvae of *A. simplex* and *P. decipiens* recovered from Atlantic cod and herring, *Clupea harengus harengus*. All nematode specimens were frozen ( $-20^{\circ}\text{C}$ ) in 0.8% saline for at least 48 hr, then thawed. During each trial known numbers of nematodes were placed in the food processor with apparently worm-free cod fillets and processed in the manner described above by an individual unaware of the number of nematodes added. Seven trials involving 98 *A. simplex* and 7 trials involving 102 *P. decipiens* were conducted. Because worms were often accidentally cut during filleting, 2 additional trials were conducted with 16 fragments of *A. simplex* and 16 fragments of *P. decipiens* (4–10 mm in length). All nematodes recovered were identified and subsamples of 20 of each species were examined under a compound microscope to determine the effects of the treatment on the general condition of the worms.

To provide material for comparing the percentages of the total number of nematodes found by candling and slicing, followed by the new technique, freshly caught cod were collected off Har-

bour Breton, southern Newfoundland in January 1987. The mean fork length  $\pm$  SD of the fish was  $52.5 \pm 4.7$  cm (range 39–64). The musculature was removed and frozen ( $-20^{\circ}\text{C}$ ) in labelled plastic bags for 3 mo. The flesh was thawed and skinned, and the nape portion separated from the fillet. Fillets and napes were examined separately for nematodes on a candling light and the musculature was sliced into thin (1.5 cm) strips to reveal nematodes deeply embedded in the flesh. Nematodes were removed and preserved in glycerin–alcohol. The flesh was reexamined using the new technique described above and additional nematodes removed, identified, and preserved.

The blind trials gave overall percentage recoveries of 98.9 and 100 for whole *A. simplex* and *P. decipiens*, respectively, and 100% for worm fragments, indicating that the new technique can recover almost all of the nematodes added. All whole specimens were recovered intact, except a single *A. simplex* that had a damaged cuticle. Examination under a compound microscope ( $\times 100$ ) revealed that although most of the specimens showed damage to the intestine, they were in otherwise good condition and easily identifiable.

Candling and slicing revealed many nematodes in the musculature of the sample of cod, but reinspection of the flesh using the new technique indicated that a substantial proportion of the *A. simplex* (30.1%) and some *P. decipiens* (4.9%) had been overlooked (Table I). The new technique was particularly effective for recovering *A. simplex* from the thicker fillet portion, where only 42.6% was detected by candling and slicing.

The results clearly demonstrate that the present technique offers an accurate and relatively rapid means of finding larval *A. simplex* and *P. decipiens* in the flesh of cod. Preliminary trials with other gadoids, salmonids, and pleuronectids indicate that the technique can be used with similar efficiency on many other marine fish species. Species such as herring, *Clupea* spp., may be exceptions in that they have bony, oily flesh and may not homogenize well. The technique has not been tested for recovering other nematode species or other parasites (e.g., larval cestodes, diagenans, acanthocephalans) from the flesh.

Pippy (1970) showed that UV light can also be used to enhance detection of larval ascaridoid nematodes in the body cavity and viscera of fish, particularly if the fish have been frozen and thawed. Under normal (white) light the nematodes are difficult to see against the host's tissues;

however, under UV light they fluoresce brightly and are quickly and easily detected. The UV light can be used on fish viscera to detect larvae of *A. simplex*, *P. decipiens*, *Contracaecum osculatatum*, *Phocascaris* spp., and *Hysterothylacium* (= *Thynnascaris*) spp., all of which fluoresce under UV light after freezing and thawing. Thus, the mechanical disintegration technique described above can be combined with inspection of the viscera under UV light to yield rapid and accurate counts of these larval ascaridoids from whole fish.

I thank G. Chaput and J. L. Andrews of LGL Ltd. for diligently processing the sample of cod, and W. Edison for technical assistance. Glenys Hughes and Drs. J. H. C. Pippy and J. W. Smith provided helpful comments on the manuscript.

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## On the Status of *Eimeria nieschulzi* Oocysts Embedded in Resin Eleven Years Ago: A Permanent Method for Preserving Coccidian Oocysts

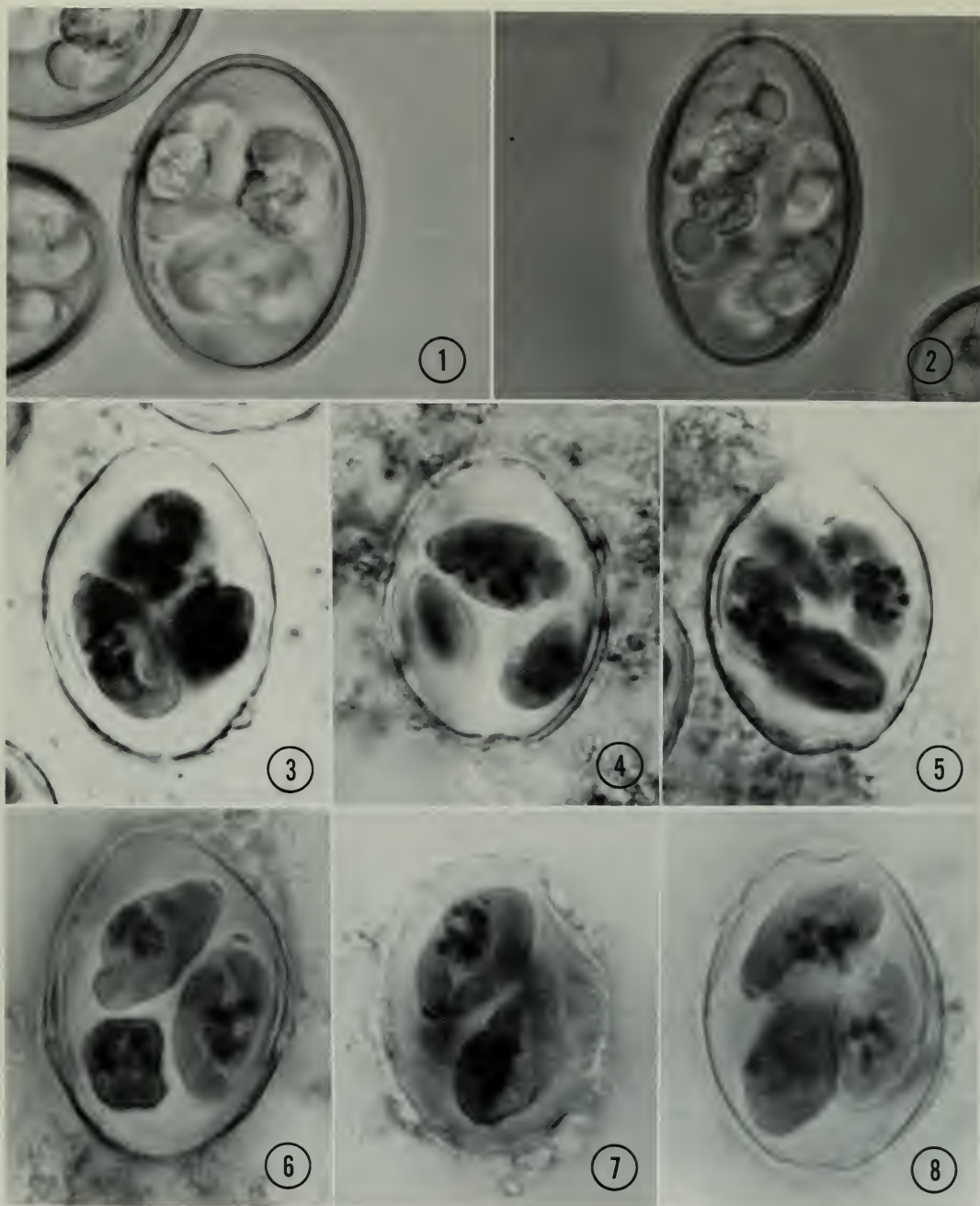
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**ABSTRACT:** Sporulated oocysts of *Eimeria nieschulzi* that were fixed and mounted on glass slides in polymerized resin in 1976 are examined. Size, shape, and integrity of oocysts and sporocysts are compared to similar observations we made in 1977 and reported in 1978 (*Journal of Parasitology* 64: 163-164). Our conclusion is that the methods we reported on in 1978 provide one opportunity to produce permanent specimens of sporulated oocysts that could be made available for deposit in nationally accredited museums.

In 1978 we reported 2 methods to produce

permanent slides of coccidian oocysts (Marchiondo and Duszynski, 1978) for light microscopy. Bandoni and Duszynski (1988) discuss the problems created in coccidian systematic studies by the failure of workers in the discipline to adhere to a type tradition when describing new species of coccidia from sporulated oocysts. They note that our preservation techniques and the use of good photomicrographs could help preserve key oocyst structures for later identification





FIGURES 1-8. Photomicrographs of sporulated oocysts of *Eimeria nieschulzi*.  $\times 1,800$ . 1, 2. Live oocysts showing some of the variation in shape seen in normal populations of *E. nieschulzi*. 3-5. Prints of negatives of oocysts embedded in resin (see Marchiondo and Duszynski, 1978) and photographed in 1977, about 15 mo after oocysts were mounted and polymerization occurred. 6-8. Oocysts on the same slide as oocysts in Figures 3-5, but photographed in 1987, 11 yr after mounting and polymerization.

and that the effort to use such techniques by those who describe new species based on oocyst structure can help remedy the current lack of a type tradition in coccidian systematics. Thus, it

seemed appropriate at this time to reexamine the slides we prepared more than a decade ago to determine whether or not the oocysts mounted on them have maintained their structural integ-

TABLE I. Measurements of sporulated oocysts of *Eimeria nieschulzi* in 3 different groups: 2 batches of live oocysts of different ages; oocysts fixed, embedded, and mounted by 2 different techniques and measured in 1977; oocysts on the same slides as in 1977, but measured 10 yr later.\*

Oocyst groups	Oocyst			Sporocyst		
	Length	Width	L/W	Length	Width	L/W
Live oocysts						
4 mo old	22.1 (20–25)	17.2 (15–18)	1.3	11.9 (10–13)	7.9 (7–9)	1.5
40 mo old	22.5 (20–26)	18.2 (16–20)	1.2	12.4 (10–15)	7.8 (7–9)	1.6
1977 oocysts†						
kf-043-1‡	22.4 (20–25)	17.0 (14–20)	1.3	11.0 (9–13)	6.4 (6–8)	1.7
kf-063-2§	21.8 (19–24)	16.9 (15–20)	1.3	10.4 (9–12)	6.3 (6–8)	1.65
1987 oocysts						
kf-043-1	23.3 (20–26)	17.5 (16–19)	1.3	11.1 (9–12)	6.4 (5–8)	1.7
kf-063-2	23.0 (19–26)	17.6 (15–19)	1.3	11.5 (9–13)	7.4 (6–8)	1.55

\* All measurements are means (n = 25) followed by the (ranges).

† Oocysts measured 15 mo after treatment/affixation to slides (see Marchiondo and Duszynski, 1978).

‡ Treatment group: CO<sub>2</sub> (5%), glutaraldehyde (15%), OsO<sub>4</sub> (2%), Epon 812.

§ Treatment group: CO<sub>2</sub> (5%), Karnovsky's, OsO<sub>4</sub> (2%), Spurr's.

urity. Table I and Figures 1–8 show the results of our study of these preserved oocysts. Although there was some initial shrinkage of oocysts during the first year after embedding, probable compression of oocysts under the cover glass over time has caused the oocysts to swell slightly in the decade since they were last measured (Table I). Nonetheless, many oocysts on our original slides are still structurally intact and are as clearly visible after 11 years' preservation as when initially embedded (cf. Figs. 3–5 vs. 6–8).

When descriptions of new coccidians are presented to journals for review and publication, editors may one day require, as they do for descriptions of new helminth species, that types

first be on deposit with nationally accredited museums. The methods we presented earlier (Marchiondo and Duszynski, 1978) may then be useful for those who work with oocysts as one way to produce type specimens (or at least syntypes) of coccidian oocysts.

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## CRITICAL COMMENT . . .

### Predation of Ticks by Shrews

I would like to point out an error in the otherwise well prepared and stimulating article by L. M. Cooksey and R. B. Davey, "Predation on cattle fever ticks . . ." (1987, *Journal of Parasitology* 73: 1272-1273). In this contribution, it is stated that "Shrews (*Crocidura* spp.) have been known to prey on engorged female *Ixodes ricinus* in Britain (Milne, 1950, *Parasitology* 40: 14-34) . . ." However, no *Crocidura* sp. shrews are native to mainland Britain (some small offshore islands to the south and west of England are inhabited by *Crocidura* shrews). The Milne study mentioned was undertaken

at 2 sites in Northumberland in northern England, and although the predatory shrews in this study were principally referred to as "common shrews," these were also stated to be *Sorex araneus* (a widespread and common insectivore throughout England, Wales, and Scotland but absent from Ireland).

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## ANNOUNCEMENT . . .

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## In Memoriam

H. E. SHORTT

15 April 1887–9 November 1987

H. E. Shortt was born in Dhari Wall in the Punjab, India, and went to school at the Inverness Academy, staying with his grandmother while his parents remained in India. Shortt went to Aberdeen University where he played hockey and was selected to play for Scotland, but had, instead, to leave for India. He passed the exams for the Indian Medical Service, and in 1911 he left for India to take up temporary duties in Lucknow and promptly took his gun out into the country to bag some partridges and pigeon. He confesses to a life-long passion for the gun, the rifle, and the rod. His first posting proper was to Benares as medical officer to the 62nd Punjabis.

At the outbreak of the First World War he accompanied the Mesopotamian Expeditionary Force to Basra in present-day Iraq. He met Sister Eleanor Hobson, Hobby to all, in Iraq, proposed in a hut in front of a wolf, and eventually married her in Calcutta in 1921.

In 1919 he was posted back to Kasauli Central Research Institute, where he met Patton, Craighead, Barraud, and Hugh Mulligan, a life-long friend, and met another friend, Ricard Christophers, who was in charge of the next-door Malaria Bureau, where Sinton also worked. Here he settled down to medical protozoology and the more serious business of fishing. The former started him off on a long study of Kala-Azar, which he commenced to follow in Assam in 1924 with Christophers, Barraud, and Craighead. Here he finally showed that sandflies were the vectors of Kala-Azar.

From Assam he returned to Kasauli in 1931 to become director of the Pasteur Institute. In 1935 he was appointed director of the King Institute. He was awarded the degrees of M.D. and D.Sc. at Aberdeen University. In 1939 he accepted a post as protozoologist to the London School of Hygiene and Tropical Medicine and he left India and the Indian Medical Service. However, with the outbreak of war he was promptly posted back to India, the IMS, and the King Institute.

War with Japan broke out and he was posted as full colonel to Assam as Inspector-General of Hospitals and Prisons and lived and worked through the attempted Japanese invasion of Assam. In 1941 he was awarded the Order of Companion of the Indian Empire, and in 1945 the Kaiser-I-Hind medal for services to the government of India. In Assam he was responsible for the erection, maintenance, and holding of a line of camps in the jungle to accommodate refugees from Burma. These camps housed temporally over one-quarter of a million transients during the Japanese advance. In 1945 he returned to take up his post at the London School of Hygiene and Tropical Medicine, where as professor of Medical Protozoology he took

over the Department of Parasitology. P. C. C. Garnham joined him as Reader and both commenced work that resulted in the discovery of the tissue stage of malaria parasites.

In 1949 he was awarded the Mary Kingsley Prize and in 1950 he was made fellow of the Royal Society. Shortt retired in 1951 only to go to Dacca to set up an Institute of Public Health and a Pathology Department. He was awarded the Darling Prize and Medal, the Gaspar Vianna Medal, and the BMA's Stewart Prize in 1952. On his return he went on working with *Babesia*, work that culminated in a visit to Nairobi in 1970 when he was only 84, for further work on *Babesia* in cattle.

In 1971, at age 85, he finally decided to call it a day for protozoology. His real interests remained, as always, shooting and fishing. He gave up rough shooting reluctantly when he was 96, and at 100 he fished every week of the season, catching his limit of trout without fail.

The dominant themes in Col. Shortt's life were love of the hunt and the outdoors. When he could combine these with medical protozoology, as in the proof of the ability of *Phlebotomus argentipes* to transmit Kala-Azar in Assam or the rediscovery of *Plasmodium galinaceum* in the jungle fowl of Madras State, he was at his best and happiest.

He was a master of the gun, the rod, and the microscope, knowing the use of each as few others have done. All the patience and tenacity required to rid an Indian village of a cattle- or man-eating tiger would also chase down and reveal the exoerythrocytic forms of primate malaria parasites in the liver.

Shortt was a hunter, happiest in the jungle, but at home in the laboratory. At the time of his 100th birthday he had both caught his usual quota of trout at his beloved Two Lakes on the sacrosanct Tuesday morning and summoned a previous assistant to instruct him to conduct an experiment that would resolve the question of the hypnozoite versus the reinventing merozoite as a cause of malarial relapse.

Shortt was also a master technician. He tied his own flies, infuriating my long-sighted wife by removing his glasses to do so. He invented his own fixatives and staining techniques. Try as we might, we never fooled him with various tricks under a microscope. His word, once at the microscope seat, was absolute law.

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R. S. Bray, Imperial College Field Station, Silwood Park, Ashurst Lodge, Ascot, Berkshire SL3 7DF, United Kingdom.



## In Memoriam

### FRANCIS J. KRUIDENIER

### 1913–1988

Francis J. Kruidenier, 75, died 16 April 1988 in Urbana, Illinois. He was born 28 June 1913 in Cedar Rapids, Iowa. He received his B.S. (1936), M.S. (1938), and Ph.D. (1951) degrees from the University of Michigan, where he was a student of Dr. George R. LaRue. After being an assistant and fellow at the University of Michigan, he came to the University of Illinois in 1948 as an instructor, and rose through the ranks to full professor in 1961. He was a member of the Department of Zoology staff and remained there, teaching general parasitology, until his retirement in 1983. He was especially interested in trematodes and other helminths, although he also did a little work on protozoa. His research was in electron microscopy, histochemistry, and immunology.

Fran was a fine teacher and a careful, reliable, and industrious research worker. He did a great deal of both field and laboratory work, but published less than he would have if he had been less thorough. He was an inspiration to everyone who worked with or under him. He was a heavy cigarette smoker, and had emphysema for many years before his death.

Fran was the principal organizer of the World Federation of Parasitologists, and was its Secretary from 1960–1971. He was Secretary of the American Society of Parasitologists from 1960–1962, its Secretary-Treasurer from 1963–1965, and its Vice-President in 1966. He was also a member of the Electron Microscope Society of America, the American Association for the Advancement of Science, the American Microscopical Society and the American Society of Zoologists.

Fran is survived by his wife Hermie of Urbana, Illinois; a sister, Mrs. Eunice Gauss of Bloomfield Hills, Michigan; six sons, Bastian of Aylmer, Quebec, Canada, John of Cambridge, Massachusetts, James of Olympia, Washington, Daniel of Thousand Oaks, California, William of Champaign, Illinois, Richard of Carr, Colorado; and seven grandchildren.

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**Norman D. Levine**, College of Veterinary Medicine, University of Illinois, Urbana, Illinois 61801.

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## MICROFILARIAL SURFACE CARBOHYDRATES AS A FUNCTION OF DEVELOPMENTAL STAGE AND ENSHEATHMENT STATUS IN SIX SPECIES OF FILARIIDS

C. W. Paulson, R. H. Jacobson\*, and E. W. Cupp†

Department of Biology, Concordia College, Moorhead, Minnesota 56560

**ABSTRACT:** The lectin-binding properties of microfilariae of *Onchocerca volvulus*, *O. lienalis*, *Brugia pahangi*, *Wuchereria bancrofti*, *Dirofilaria immitis*, and *Monanema* (= *Ackertia*) *marmotae* share a number of characteristics. Carbohydrates specific for lectins are associated with the egg shell or sheath. N-acetyl-D-glucosamine is the predominant carbohydrate associated with the ensheathed forms with lesser quantities of D-galactose and/or  $\alpha$ -lactose and D-galactosamine. The density of these carbohydrates on the sheath surface diminishes as the larvae undergo normal growth and development. Similar carbohydrates are not found on the cuticle as exsheathed microfilariae show virtually no ability to bind lectins.

Because the surface of a parasite interacts directly with body fluids and tissues of the host, an analysis of surface composition is of significance in elucidating its immunological function. Numerous studies have focussed on antigenic properties of the surface of parasitic nematodes (Mackenzie et al., 1978; Murrell and Graham, 1982; Jungery et al., 1983) with emphasis placed on polypeptide or protein antigens (Philipp et al., 1980; Forsyth et al., 1981; Maizels et al., 1983a, 1983b, 1983c).

Recently, considerable interest has developed in carbohydrate antigenicity and composition of parasite surfaces. Furman and Ash (1983a) have suggested that sheath carbohydrate antigenic determinants may be the primary targets of the host's immune response. Lectin-binding studies have demonstrated specific saccharides on the surface of the filariid nematodes, *Brugia pahangi* (Furman and Ash, 1983a, 1983b; Devaney, 1985), *Brugia malayi* (Kaushal et al., 1984), *Onchocerca gutturosa* (Nwachukwu et al., 1987), and *Wuchereria bancrofti* (Rao et al., 1987). The saccharide residues present may be stage as well as species specific (Furman and Ash, 1983a, 1983b; Kaushal et al., 1984).

Among filarial worms, microfilariae of certain species retain the sheath (egg membrane) following hatching. In other species, microfilariae hatch and leave the sheath *in utero*, thereby living in the host's tissues unsheathed. Because the sheath has implications for the immunogenicity of this

stage (see Hammerberg et al., 1984), this study was designed to compare surface carbohydrate patterns of sheathed (*Monanema marmotae*, *B. pahangi*, and *Wuchereria bancrofti*) and unsheathed species (*Dirofilaria immitis*, *Onchocerca volvulus*, and *O. lienalis*). To determine whether carbohydrate surface components were stage as well as species specific, lectin-binding properties associated with *in utero* larval development were also examined in 3 species, *M. marmotae*, *B. pahangi*, and *O. volvulus*.

### MATERIALS AND METHODS

#### Sources of parasites

Adults and microfilariae (mf) of *Monanema marmotae* were recovered from woodchucks (*Marmota monax*) trapped in Tompkins and Seneca counties, New York. Adults were recovered from the lymphatic vessels of adipose tissue surrounding the gall bladder and bile ducts (Ko, 1972). Mature females were placed into a 2-ml glass tissue homogenizer containing phosphate-buffered saline (PBS), pH 7.4, and disrupted by gentle action to release the developing eggs and larval stages. Eggs and larvae were sedimented by centrifugation (225 g for 5 min) and then resuspended in PBS. The process was repeated as needed to remove extraneous material.

Skin-derived mf of *M. marmotae* were recovered from the woodchuck's ears that were minced, placed in PBS, and incubated 4 hr at 37 C. Following incubation, the mixture was filtered through cheesecloth to remove remaining tissue and the mf were recovered by centrifugation and washing as described above.

Larvae were generally utilized in lectin-binding studies within a few hours of recovery; however, in a few cases worms were held for several days at 5 C in 0.1% buffered formalin (Kaushal et al., 1984).

Early developmental stages and mature mf of *O. volvulus* were recovered from nodular or skin snip samples, respectively, from Guatemala. Nodules or skin snips were cut into small pieces and maintained frozen in liquid nitrogen until ready for use. Samples were thawed in a waterbath at 37 C, placed in 10 ml Medium

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199 (GIBCO Laboratories, Grand Island, New York) containing 20% (v/v) of fetal calf serum and incubated at 37 C for 2 hr. These larvae (still viable and active) were centrifuged at 225 g for 5 min, washed twice with 10 ml PBS, and resuspended in a small quantity of PBS.

*Onchocerca lienalis* mf were recovered from umbilical skin of cattle obtained locally (Tompkins County, New York), cryopreserved, and held in liquid nitrogen (Lok et al., 1980). Prior to lectin exposure, mf were thawed, washed, and resuspended in PBS as described above.

Adults and mf of *Brugia pahangi* were recovered from the peritoneal cavity of jirds supplied by Dr. John McCall, University of Georgia. Microfilariae were harvested by lavage using PBS followed by centrifugation at 225 g and resuspension in PBS. Mature females were washed in PBS, placed in a 2-ml glass tissue homogenizer, and disrupted by gentle action to release *in utero* larval stages.

Microfilariae of *Dirofilaria immitis* were recovered from blood by a modification of the method of Sawyer and Weinstein (1963). Blood was diluted (1:11) in 0.2% saponin with 0.85% NaCl in distilled water, incubated at 37 C for 15 min followed by centrifugation at 225 g for 10 min. The resulting pellet was suspended in 10 ml PBS, incubated at room temperature for 10 min, and centrifuged. The washing procedure was repeated and the final pellet resuspended in 1 ml PBS.

*Wuchereria bancrofti* mf were recovered from the cryopreserved blood of infected persons in Liberia. The samples, maintained in liquid nitrogen for approximately 2 yr, were thawed in a waterbath at 37 C. They were suspended in PBS (1:11) and incubated at 37 C for 10 min followed by centrifugation at 225 g for 10 min. Following saponin treatment as described above, they were washed and resuspended in PBS.

### Lectin binding

Fluorescein-labeled lectins and their saccharide inhibitors were all purchased from Sigma Chemical Company, St. Louis, Missouri. These included concanavalin A (Con A), primarily specific for D-mannose and  $\alpha$ -methyl-D-mannoside; *Glycine max* (soybean) agglutinin type VI (SBA), specific for N-acetyl-D-galactosamine; peanut agglutinin (PNA), specific for D-galactose; *Ricinus communis* agglutinins (RCA-I), specific for D-galactose and  $\alpha$ -lactose; (RCA-II), specific for D-galactose; *Ulex europaeus* agglutinin (UEA-I), specific for  $\alpha$ -L-fucose; and wheat germ agglutinin (WGA), specific for N-acetyl-D-glucosamine.

The lectin-binding assay was a modification of that described by Kaushal et al. (1984). Known numbers of worms were placed in PBS containing lectin (100  $\mu$ g/ml) in a total volume of 200  $\mu$ l. The appropriate competing sugar was added to a corresponding tube at a final concentration of 150 mM as a check on specificity of the binding reaction. Incubation was carried out at room temperature for 30 min in the dark and was followed by 3 cycles of washing in PBS (4 C) and centrifugation at 225 g for 5 min. An aliquot was then placed on a glass slide and the bound fluorescence measured on individual parasites using a Nikon Microflex UFX photomicrographic attachment on a Leitz Ortholux fluorescent microscope. Numerical readings (in-

dicating the required photographic exposure time) were converted into fluorescence units defined as the reciprocal of the numerical reading  $\times$  1,000. Fluorescence units were thus a direct correlate of the quantity of lectin bound by the parasite. Single readings were taken on 10 individual parasites to establish mean values. Specific binding attributable to the presence of specific saccharides was determined by subtracting nonspecific binding (binding with specific saccharide inhibitor present) from total binding (no inhibitor present).

### Exsheathment

Microfilariae of *B. pahangi* were exsheathed according to the method of Devaney and Howells (1979) using papain (Type III, Sigma Chemical Co., St. Louis, Missouri) at 5 units/ml. A small percentage of *M. marmotae* mf had already lost the sheath when recovered from ear tissue and so *in vitro* exsheathment procedures were not required.

## RESULTS

*In utero*-derived larval stages of 3 species (*Onchocerca volvulus*, *Monanema marmotae*, and *Brugia pahangi*) were exposed to a panel of 7 lectins to determine whether specific carbohydrates were associated with the surface and whether these might be stage and species specific. For comparative purposes, these forms were subdivided as follows: "early egg (nonembryonated) stages," designated as those that were clearly in an early stage of embryonic development, i.e., prior to elongation into the vermiform shape; as "larvated eggs," those containing a well-developed vermiform stage but still having a spherical or slightly ovoid configuration; "*in utero*-derived mf," those fully developed larval forms uncoiled within the stretched and greatly elongated egg shell membrane sheath.

### *Onchocerca volvulus*

Microfilariae of this species lose their sheath *in utero*; thus, only 2 stages (larvated eggs and "exsheathed" mf) could be readily identified and recovered.

Larvated eggs bound all lectins tested (Table I). PNA, RCA-I, RCA-II, and SBA bound with high specificity and could be readily inhibited (72–95%) by the addition of competing sugars at concentrations of 150 mM. WGA bound to the larvated egg at greater levels than other lectins tested; however, only a small portion of the binding (12%) was prevented by the presence of 150 mM N-acetyl-D-glucosamine.

All lectin binding of *in utero*-derived *O. volvulus* larvae was associated with the sheath because hatched mf showed no ability to bind any of the lectins tested.

TABLE I. Binding of lectins by in utero-derived larvated eggs of *Onchocerca volvulus*.

Lectin	Major specificity	Relative fluorescence			
		Total binding	Binding in the presence of 150 mM inhibitor added	Specific binding	Percent inhibition
Con A	D-mannose; $\alpha$ -methyl-D-mannoside	5.2 $\pm$ 1.6*	3.3 $\pm$ 1.1*	1.9†	37
WGA	N-acetyl-D-glucosamine	117.0 $\pm$ 18.8	102.4 $\pm$ 16.0	14.6	12
PNA	D-galactose	24.4 $\pm$ 13.1	3.7 $\pm$ 1.1	20.7	85
RCA-I	D-galactose; $\alpha$ -lactose	69.1 $\pm$ 17.1	19.6 $\pm$ 7.4	49.5	72
RCA-II	D-galactose	87.9 $\pm$ 18.4	12.6 $\pm$ 3.5	75.3	86
SBA	N-acetyl-D-galactosamine	93.5 $\pm$ 32.6	4.4 $\pm$ 0.7	89.1	95
UEA-I	$\alpha$ -L-fucose	10.1 $\pm$ 2.7	11.5 $\pm$ 2.4	0	0

\* Mean  $\pm$  SD of 10 observations.

† Total binding minus binding in the presence of specific inhibitor.

Skin snip-derived mf also bound no lectins; thus maturation and migration through the host tissues had no apparent effect on surface carbohydrate composition.

### *Monanema marmotae*

Marked differences were found in the surface carbohydrate composition of the various larval stages of *M. marmotae* (Table II). Early egg stages bound the greatest variety of lectins although the density of bound lectins (intensity of fluorescence observed) was comparatively low. Generally as *in utero* larval development progressed to the larvated egg stage, the intensity of lectin binding increased dramatically with the highest levels of fluorescence being observed at this time. Further morphogenesis and development to the microfilarial form led to decreases in both diversity and density of lectins bound.

Skin-derived mf bound WGA and only slightly bound RCA-I, i.e., fluorescence associated with RCA-I was visible on the sheath but measurements of intensity were only slightly above back-

ground levels. Microfilariae that had lost their sheath were unable to bind any of the lectins tested.

WGA bound to all larval stages at greater levels than all other lectins tested. This binding was inhibited by the addition of N-acetyl-D-glucosamine thus demonstrating the specificity of WGA for this moiety.

On a few occasions worms were fixed in 0.1% buffered formalin and held overnight prior to lectin exposure. To ensure that this fixation did not alter binding properties, the binding of WGA to fixed mf was compared with that to live worms. An equivalent level of binding was observed in each case.

### *Brugia pahangi*

Developing larval stages of *B. pahangi* bound lectins in a pattern somewhat similar to that observed for *M. marmotae*, i.e., larvated eggs strongly bound WGA and demonstrated a lesser affinity for PNA, RCA-I, RCA-II, and SBA (Table III). Additionally, the quantity as well as the diversity of lectins bound decreased dramati-

TABLE II. Binding of lectins by larval stages of *Monanema marmotae*.

Lectin	Relative fluorescence*				
	<i>In utero</i> stages			Skin-derived microfilariae	
	Non-embryonated egg	Larvated egg	Microfilaria	Sheathed	Ex-sheathed
ConA	3.0	4.7	<1	<1	<1
WGA	17.1	207.6	41.6	18.2	<1
PNA	1.6	<1	<1	<1	<1
RCA-I	8.7	66.6	11.2	1.5	<1
RCA-II	9.4	23.8	<1	<1	<1
SBA	12.3	46.1	3.3	<1	<1
UEA	<1	<1	<1	<1	<1

\* Each value represents specific binding (mean total binding [ $n = 10$ ] minus mean binding in the presence of 150 mM of specific saccharide inhibitor [ $n = 10$ ]).TABLE III. Binding of lectins by larval stages of *Brugia pahangi*.

Lectin	Relative fluorescence*			
	<i>In utero</i> -derived		Peritoneal microfilariae	
	Larvated eggs	Microfilariae	Sheathed	Ex-sheathed
Con A	<1	<1	<1	<1
WGA	517	37	29	<1
PNA	5	<1	<1	<1
RCA-I	59	6	<1	<1
RCA-II	12	<1	<1	<1
SBA	37	<1	<1	<1
UEA	<1	<1	<1	<1

\* Each value represents specific binding (mean total binding [ $n = 10$ ] minus mean binding in the presence of 150 mM of specific saccharide inhibitor [ $n = 10$ ]).



cally to the typical microfilarial form as the larva matured *in utero*. Peritoneal mf bound only WGA and none of the lectins were bound by the parasites following exsheathment. Some species specificity was evident as larvated eggs of *B. pahangi* bound a low level of PNA but did not bind Con A in contrast to those of *M. marmotae* that bound Con A but not PNA.

#### Other filariids

WGA bound strongly to the ensheathed mf of *W. bancrofti*, whereas a low level of binding was associated with RCA-I. None of the panel of 7 lectins was bound by the mf (unsheathed) of *D. immitis* or *O. lienalis*.

### DISCUSSION

Lectin binding by the larval stages of 6 species of filarial worms was associated with the sheath of the organism but was not characteristic of the exsheathed mf. These studies show that the sheaths surrounding the early stages of *B. pahangi*, *O. volvulus*, and *M. marmotae* embryos have on their surfaces a variety of exposed carbohydrate moieties with a predominance of N-acetyl-D-glucosamine. This is similar to the findings of Nwachukwu et al. (1987) for *O. gutturosa*. As development progresses, the variety of these sugars is decreased with the mature microfilarial sheath showing only N-acetyl-D-glucosamine and possibly D-galactose. Devaney (1985) and Rao et al. (1987) have also demonstrated that N-acetyl-D-glucosamine predominates on the surface of circulating mf of *B. pahangi* and *W. bancrofti*, respectively.

Concurrently, the quantity of lectin that will bind to the surface (although showing an initial increase during early embryonic development) declines as the parasite matures. These data are essentially in agreement with those of Furman and Ash (1983a, 1983b) who found that mf of *B. pahangi* recovered directly from adult females bound a greater variety of lectins than those recovered from the peritoneal cavity of the host. They concluded that maturation and release of mf are correlated with glycosidic alterations on the sheath surface. Kaushal et al. (1984) showed that lectin binding by *B. malayi* also varied with maturation and development as mf recovered intraperitoneally bound fewer lectins than those derived directly from the adult females.

In the present study, intraperitoneally derived mf of *B. pahangi* strongly bound WGA but showed no affinity for Con A in contrast to the

findings of Furman and Ash (1983a) who report that this stage bound both Con A and WGA.

*Monanema marmotae* (Table II) and *B. pahangi* nonembryonated eggs bound small amounts of lectins, and embryonated eggs bound lectins quite efficiently. This pattern parallels that observed by Taylor et al. (1986) who noted the existence of 2 distinct populations of eggs for *O. volvulus*.

Others (Sayers et al., 1984; George et al., 1985) have suggested that some surface antigens of several filariids are of host origin; however, the present study with developing stages of *M. marmotae* and *B. pahangi* indicates that surface carbohydrate composition is probably unrelated to exposure to serum or other tissue fluids of the host. This observation is consistent with that of Taylor et al. (1986) who found that microfilariae of *O. volvulus* did not bind serum proteins. Supporting evidence comes from our observation that carbohydrate levels are highest in larvated eggs and diminish with maturation; thus, patterns of change are well established by the time mf are expelled from the body of the female. As the larvae mature in their uterine passage, specific carbohydrates are either being removed from the sheath or the exposed molecules are being masked and are no longer available for interaction with lectins.

Because carbohydrates likely play a significant role in surface antigenicity, it appears that sheathed mf present to the host a far different array of antigens than are associated with later stages of the life cycle (following exsheathment). An immune response directed against ensheathed mf may not be effective against other stages of the parasite.

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## PRIMAQUINE IS LETHAL FOR INTRACELLULAR BUT NOT EXTRACELLULAR *TRYPANOSOMA CRUZI*

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**ABSTRACT:** Primaquine has been used to treat Chagas' disease in humans and has been reported to be active against extracellular *Trypanosoma cruzi*. Experiments were designed to evaluate the relative activity of primaquine against extra- and intracellular *T. cruzi* and to determine if primaquine might be combined advantageously with ketoconazole. Primaquine at 0.5  $\mu\text{g/ml}$  significantly inhibited *T. cruzi* replication in infected mouse peritoneal macrophages and also effectively treated infected L929 cells. To examine the effect of primaquine on extracellular organisms, tissue culture *T. cruzi* were incubated with primaquine for different periods of time and then used to infect macrophages. Incubation with 10  $\mu\text{g/ml}$  for 14 hr but not 8 hr significantly inhibited but did not eradicate replication. Incubation of spleen amastigotes or blood trypomastigotes for 2 hr with 10  $\mu\text{g/ml}$  did not inhibit replication. Incubation of extracellular tissue culture *T. cruzi* with primaquine for 2 hr did not potentiate the activity of ketoconazole against intracellular organisms. The combination of primaquine and ketoconazole administered to acutely infected mice significantly decreased parasitemias in comparison to treatment with primaquine or ketoconazole alone. Thus primaquine acts primarily on intracellular rather than extracellular *T. cruzi*. Primaquine and ketoconazole appear to have additive activity *in vivo*.

Chemotherapy for acute and chronic Chagas' disease is unsatisfactory. The agents used for human disease, nifurtimox and benznidazole, suppress rather than cure the infection, require prolonged treatment courses, and are potentially toxic (Brener, 1979; Marr and Docampo, 1986). Higher concentrations of these agents are needed to demonstrate activity against extracellular than against intracellular *Trypanosoma cruzi*, which suggests that intracellular organisms are the primary targets for these drugs (Gonnert and Bock, 1972; Brener, 1979; Richle and Raaflaub, 1980; Filardi and Brener, 1984). Imidazoles and inhibitors of purine metabolism such as allopurinol and formycin B inhibit *T. cruzi* both *in vivo* and *in vitro*, but act primarily against intracellular organisms (McCabe et al., 1984a, 1985, 1986). A drug that has marked activity against bloodstream trypomastigotes at clinically attainable concentrations may have advantages over agents that are active primarily against intracellular organisms and thus be appropriate for intensive investigation of its therapeutic potential. Drugs in current clinical use are the most obvious candidates for evaluation.

Primaquine is used extensively in humans to treat malaria, with major activity against liver schizonts. Primaquine administered orally to mice at a dose of 0.25 mg/day suppressed acute

infection with the Tulahuen strain of *T. cruzi* (Pizzi, 1951). Primaquine has been used to treat humans with laboratory-acquired and congenital *T. cruzi* infections (Howard et al., 1957; Pizzi et al., 1963). Despite use of primaquine for treatment of Chagas' disease, very few investigations of the activity of primaquine against *T. cruzi* have been performed *in vitro*. Pereira da Silva and Kirchner (1962), using tissue cultures infected with the Y strain, concluded that primaquine acted primarily against extracellular trypomastigotes on the basis of observation of decreased numbers of organisms in extracellular medium. However, these results may have been due to intracellular inhibition of development of trypomastigotes rather than to a direct effect of primaquine on extracellular trypomastigotes.

After showing in my laboratory that primaquine administered orally (0.25 mg/day) suppressed acute infection with the Y strain of *T. cruzi* in outbred Swiss-Webster mice, experiments were designed to evaluate the relative activity of primaquine against intracellular and extracellular *T. cruzi*. In addition, experiments were performed to determine if exposure of extracellular organisms to concentrations of primaquine that did not kill the organisms would potentiate the activity of ketoconazole against intracellular organisms *in vitro*. Such potentiation might be important in tissues with low concentrations of ketoconazole or when resistant strains of *T. cruzi* are treated. Ketoconazole is active primarily against intracellular *T. cruzi* (McCabe et al.,

1984a) and is used widely for treatment of fungal infections in humans.

## MATERIALS AND METHODS

### *Trypanosoma cruzi*

The Y strain (Melo and Brener, 1978) was used in all experiments. In most experiments, organisms were obtained from infected cultures of L929 cells as previously described (Araujo and Remington, 1981). These preparations usually contained about 90% amastigotes and 10% trypomastigotes. In some experiments blood-form trypomastigotes and spleen amastigotes were obtained from Swiss-Webster mice (Bantin and Kingman, Fremont, California) infected for 7 days with the Y strain, by previously described methods (McCabe et al., 1984a, 1984b). Epimastigotes were cultured as described previously (McCabe et al., 1984a). For *in vivo* experiments, mice were infected by intraperitoneal injection with blood-form trypomastigotes from acutely infected mice.

### Infection of macrophages and L929 cells

Monolayers of resident mouse peritoneal macrophages were established in 8-chamber tissue culture slides (Labtek Products, Naperville, Illinois) (McCabe et al., 1984a). In some experiments monolayers of L929 cells, a mouse subcutaneous fibroblast line, were used. In the usual experiment, *T. cruzi* were added to the monolayers at a parasite-to-cell ratio of approximately 1–2:1 and incubated for 2 hr. At that time, extracellular organisms were removed by extensive washing, and the monolayers were incubated for 48 hr more. Then the monolayers were fixed with methanol, stained with 5% giemsa (Harleco, Gibbstown, New Jersey), and examined for number of infected cells and the number of organisms per infected cell (McCabe et al., 1984a).

### Drugs and reagents

Primaquine diphosphate powder was obtained from Sigma Chemical Co. (St. Louis, Missouri). Primaquine was dissolved in water at a concentration of 10 mg/ml and then diluted in medium to the desired concentration. In most experiments, *T. cruzi* were incubated with primaquine for 2 hr. This time period was chosen to approximate length of exposure of *T. cruzi* in serum to drug at peak serum concentrations (Mihaly et al., 1984). Ketoconazole powder (Janssen Pharmaceutica, New Brunswick, New Jersey) was dissolved in methanol to a stock concentration of 10 mg/ml and then diluted to the desired concentration in medium. All cultures were treated with medium that contained the same amount of methanol. RPMI-1640 tissue culture medium (GIBCO Laboratories, Grand Island, New York) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Hyclone, Logan, Utah), penicillin (100 U/ml) (Sigma), and streptomycin (100 µg/ml) (Sigma) was used in all experiments.

For *in vivo* experiments, ketoconazole was dissolved in acidified water (McCabe et al., 1984a) and primaquine was dissolved in water and administered to mice by gavage at dosages of 15 mg/kg/day and 0.25 mg/day, respectively. Mice infected with *T. cruzi* were treated with cyclophosphamide (Sigma) at a dose of 150 mg/kg by intraperitoneal injection 2 days before and after infection.

### Statistics

Student's *t*-test was used for statistical analysis, with  $P < 0.05$  considered significant. Experiments were done in duplicate, and results representative of 3 experiments are presented unless stated otherwise.

## RESULTS

The first set of experiments established that primaquine at concentrations as low as 0.5 µg/ml markedly inhibits intracellular replication of *T. cruzi* in an *in vitro* model of intracellular infection (Fig. 1). Primaquine almost sterilized the monolayer after 10 days of incubation (Fig. 1) at a concentration of 1 µg/ml, whereas a concentration of 0.1 µg/ml was minimally effective. At 10 days of infection, control monolayers were completely destroyed by infection. Similar results were observed when numbers of amastigotes per infected macrophage were analyzed (data not shown). Parallel experiments were performed with L929 cells. Higher concentrations of primaquine were needed to significantly inhibit replication of intracellular *T. cruzi* in L929 cells than in macrophages. After 48 hr of infection, treatment of infected L929 cells with 10, 5, 1, and 0 µg/ml of primaquine resulted in 0.8, 3.4, 13.4, and 20.6% infected cells, respectively, and 2.4, 5.2, 9.2, and 9.2 organisms per infected cell, respectively. The differences were statistically significant with respect to control for both percent cells infected and number of organisms per infected cell for treatment with 5 and 10 µg/ml of primaquine only.

To screen for an effect of primaquine on extracellular *T. cruzi* and to compare its effect on intracellular organisms, primaquine (5 µg/ml) was added to macrophage monolayers for 2 hr immediately before the 2-hr incubation with *T. cruzi*, for 2 hr after incubation with *T. cruzi*, or during the incubation with *T. cruzi*. The results (Table I) show that exposure of extracellular *T. cruzi* to primaquine did not enhance inhibition of replication in this protocol. Of interest, exposure of macrophages to primaquine only before infection inhibited replication of *T. cruzi* somewhat.

The foregoing experiments established that primaquine markedly inhibits intracellular replication of *T. cruzi* and suggested that activity against extracellular *T. cruzi* was considerably less. To further explore the effect of primaquine on extracellular *T. cruzi*, tissue culture *T. cruzi* were incubated with primaquine at concentrations up to 10 µg/ml for 2 and 4 hr, washed to



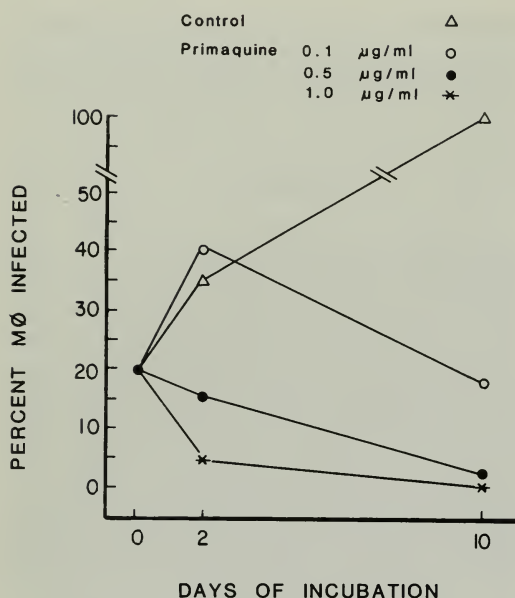


FIGURE 1. Effect of continuous exposure of infected macrophages to primaquine. Resident peritoneal macrophage monolayers were incubated with tissue culture *Trypanosoma cruzi* for 2 hr, extracellular organisms were removed by washing, and the monolayers were then incubated with primaquine for the indicated number of days.

remove primaquine, and used to infect macrophage monolayers. No effect of primaquine on either number of macrophages infected or number of organisms per infected macrophage was observed. Monolayers infected with organisms exposed to 10 µg/ml of primaquine for 4 hr had 42.0% cells infected versus 33.6% for controls, and infected macrophages contained an average

TABLE I. Effect of exposure of macrophages to primaquine for 2 hr before, during, and after incubation with *Trypanosoma cruzi*.

Primaquine treatment*	Macrophages infected (%) ± SEM	Organisms per infected macrophage ± SEM
Nontreated control	18.6 ± 1.3	6.9 ± 0.4
Before	17.6 ± 1.2	3.8 ± 0.3†
During	18.2 ± 1.0	4.3 ± 0.1†
After	13.4 ± 1.8	1.4 ± 0.1†‡

\* Peritoneal macrophage monolayers were incubated for 2 hr with tissue culture *T. cruzi*. Extracellular organisms were then removed by washing. Macrophages were incubated with primaquine (5 µg/ml) for 2 hr before, during, or after the infection period. Primaquine was removed by extensive washing. Monolayers were then incubated for 48 hr more, when they were fixed and examined.

†  $P < 0.01$  for all treatments compared with control.

‡  $P < 0.01$  for the "after" treatment compared with the other treatment groups.

TABLE II. Effect of prolonged incubation of primaquine with tissue culture *Trypanosoma cruzi* on infection and replication in macrophages.

Incubation time (hr)*	Primaquine (µg/ml)	Macrophages infected (%) ± SEM	Organisms per infected macrophage ± SEM
8	Control	46.8 ± 0.8	5.8 ± 1.0
10		29.6 ± 10.4†	4.4 ± 0.6
14	Control	51.0 ± 6.2	5.4 ± 0.1
10		29.6 ± 2.8‡	4.2 ± 0.4‡
24	Control	36.4 ± 2.0	8.7 ± 1.1
10		4.8 ± 4.0§	3.4 ± 0.4§

\* Tissue culture *Trypanosoma cruzi* were incubated with primaquine for 8, 14, and 24 hr before they were washed 3 times and used to infect macrophage monolayers. Monolayers were examined 48 hr later.

†  $P < 0.05$  compared with control.

‡  $P < 0.02$  compared with control.

§  $P < 0.01$  compared with control.

of 9.8 and 10.6 organisms, respectively. To examine the possibility that trypomastigotes obtained from blood or amastigotes from spleens of outbred Swiss-Webster mice infected for 7 days differed from tissue culture *T. cruzi* in sensitivity to primaquine, blood trypomastigotes and spleen amastigotes were incubated with primaquine (concentrations up to 10 µg/ml) for 2 hr, washed, and used to infect macrophage monolayers. No effects of primaquine on number of infected macrophages or replication were observed (data not shown).

To determine whether incubation of *T. cruzi* with primaquine for longer than 4 hr would damage the organisms as assessed in this model, tissue culture organisms were incubated with primaquine (10 µg/ml) for 8, 14, and 24 hr before they were washed and used to infect macrophage monolayers. When monolayers were assessed after 48 hr more of incubation, treatment with primaquine decreased the number of infected macrophages as length of exposure to primaquine increased, suggesting decreased infectivity of the *T. cruzi* (Table II). However, no differences in replication of intracellular organisms were observed until 14 hr of incubation with primaquine, and replication was only inhibited, not eradicated.

The lower number of macrophages infected by primaquine-treated organisms (Table II) suggests that exposure of *T. cruzi* to primaquine might decrease infectivity for macrophages. Alternatively, the lower numbers of infected macrophages may reflect disappearance of killed organisms from the macrophages. To assess these possibilities, *T. cruzi* were incubated with 10, 1, and 0 µg/ml of primaquine for 14 hr and then

TABLE III. Effect of preincubation of *Trypanosoma cruzi* with primaquine on inhibition of replication by ketoconazole.\*

Primaquine ( $\mu\text{g/ml}$ )	Ketoconazole ( $\mu\text{g/ml}$ )	Organisms per infected macrophage $\pm$ SEM
0	0	9.4 $\pm$ 1.0
0	0.5	7.1 $\pm$ 0.5
0	1.0	4.3 $\pm$ 0.3
0	5.0	3.2 $\pm$ 0.6
10	0	8.5 $\pm$ 1.1
10	0.5	8.8 $\pm$ 1.6
10	1.0	4.4 $\pm$ 0.4
10	5.0	2.4 $\pm$ 0.1

\* Macrophage monolayers were incubated with ketoconazole for 30 min. Ketoconazole was removed by extensive washing. Tissue culture *Trypanosoma cruzi* that had been incubated with primaquine and then washed were used to infect the monolayers. Monolayers were examined 48 hr later.

added to macrophage monolayers, incubated for 1 hr, washed, stained, and examined. No significant differences among treatment groups were seen with respect to percent macrophages infected or number of organisms per infected macrophage (data not shown). Counts with trypan blue in this set of experiments and other experiments indicated more than 95% viability in all groups and did not indicate lysis of organisms.

Primaquine was incubated with epimastigotes in logarithmic growth. A concentration of 10  $\mu\text{g/ml}$  inhibited but did not stop logarithmic replication (counts were approximately half of controls at 8 days of incubation with drug), and growth with 1  $\mu\text{g/ml}$  was similar to control cultures.

The above experiments indicate that primaquine has a modest but definite effect against extracellular *T. cruzi* under conditions of high drug concentrations and prolonged exposure of organisms to drug (Table II). Experiments were then performed to examine the possibility that exposure of extracellular *T. cruzi* to primaquine might potentiate the activity of ketoconazole against intracellular *T. cruzi*. Macrophage monolayers were incubated with ketoconazole (0.5, 1.0, 5.0  $\mu\text{g/ml}$ ) for 30 min, washed, and then infected with tissue culture *T. cruzi* that had been preincubated with primaquine (10  $\mu\text{g/ml}$ ) for 2 hr. These conditions of exposure of macrophage monolayers to ketoconazole were chosen to minimize the effect of ketoconazole on replication and provide opportunity for observation of the effect of primaquine. However, no potentiation of the activity of ketoconazole by primaquine was observed in this model (Table III).

Two experiments were performed *in vivo*. In

TABLE IV. Effect of ketoconazole and primaquine treatment on parasitemias with *Trypanosoma cruzi*.\*

Treatment	Parasitemia (organisms/50 fields $\pm$ SEM)	
	Day 6	Day 7
Water	736 $\pm$ 62	1,470 $\pm$ 130
Ketoconazole	187 $\pm$ 25	815 $\pm$ 209
Primaquine	147 $\pm$ 27	325 $\pm$ 21
Primaquine and ketoconazole	59 $\pm$ 7	104 $\pm$ 23

\* Groups of 3 mice were infected with  $5 \times 10^5$  bloodform trypomastigotes and treated with cyclophosphamide. Starting 12 hr after infection, mice were treated with ketoconazole (15 mg/kg/day), primaquine (0.25 mg/day), both primaquine and ketoconazole, or water by daily gavage. Parasitemias were determined by light microscopy of blood smears, and results expressed as number of organisms in 50 fields with the 40 $\times$  ocular (high dry). For both days 6 and 7 of infection, parasitemias in mice that received both drugs were significantly lower ( $P < 0.05$ ) than in mice that received either single drug.

one, a modification of the protocol of Filardi and Brener (1984) was used to determine if primaquine had an effect on extracellular *T. cruzi*. Filardi and Brener (1984) reported dramatic reduction in parasitemias in the hours following oral administration of certain drugs to mice with patent parasitemia. Groups of 2 mice were gaged with either 1 mg of primaquine or water on the seventh day after infection with  $5 \times 10^5$  bloodform trypomastigotes. Parasitemias were determined by light microscopy (McCabe et al., 1984a) 1, 2, 5, and 7 hr after gavage. Neither significant differences nor trends were seen in levels of parasitemia between the 2 groups (data not shown).

The second experiment was designed to determine if the combination of ketoconazole and primaquine had an additive or synergistic effect on parasitemia. Four groups of 3 mice were infected with  $5 \times 10^5$  bloodform trypomastigotes and treated with cyclophosphamide. One group was treated with water, one with primaquine (0.25 mg/day), another with ketoconazole (15 mg/kg/day), and the last with both primaquine and ketoconazole with the aforementioned doses. Treatment was started 12 hr after infection and was continued to the seventh day of infection. Parasitemias were determined on days 6 and 7 of infection when parasitemias were patent. The results (Table IV) show that the combination of ketoconazole and primaquine was superior to single drug treatment.

## DISCUSSION

Data reported herein show that primaquine is very active against intracellular *T. cruzi* at con-



centrations of drug that can be attained in human serum and tissues (Mihaly et al., 1984). Primaquine decreased infectivity of extracellular *T. cruzi* for macrophages only at relatively high concentrations and after relatively long exposures (8–14 hr). Earlier investigations that used monkey heart cells infected with the Y strain (Pereira da Silva and Kirchner, 1962) suggested that primaquine damaged extracellular *T. cruzi* primarily. However, these studies were designed to determine if the drug had any inhibitory effect on infected cell cultures. The conclusion that primaquine damaged extracellular *T. cruzi* was based on the relative lack of extracellular organisms in the culture supernatant, which in light of the data reported here was most likely due to inhibition of intracellular replication and prevention of cell lysis and release of organisms into the medium.

Previous investigators have found it difficult to demonstrate that a drug has a direct effect on extracellular organisms. In one strategy, Filardi and Brener (1984) gave infected mice single doses of nifurtimox and benznidazole and observed reductions of parasitemias with repeated observations by light microscopy over the ensuing 6 hr. Due to rapidity of fall in parasitemia, they concluded that the drugs must be active against bloodstream organisms. However, Brener's group in another study (Schlemper et al., 1977) was unable to show that a number of drugs active *in vivo* were active *in vitro* against bloodstream trypomastigotes with incubations of drug with *T. cruzi* for 24 hr. In the experiments reported here, a direct effect of primaquine on extracellular organisms was indicated only when *T. cruzi* were incubated with primaquine for 8 hr or longer, which resulted in reduction of the number of infected macrophages and inhibition of replication of organisms that had infected the macrophages.

Primaquine did not exert an additive or synergistic effect by damage of extracellular organisms with ketoconazole in an *in vitro* model in which the inhibitory effect of ketoconazole was minimized. Primaquine might act in concert with nifurtimox and benznidazole, the standard agents for treatment of Chagas' disease, but these drugs were not studied. Primaquine and ketoconazole appeared to have at least an additive effect *in vivo*. In view of the *in vitro* results, the additive effect is most likely due to activity against intracellular organisms.

Refractoriness to chemotherapy may result from different sensitivities of different types of

cells to drugs. Primaquine was found in this report to be effective at 5  $\mu\text{g}/\text{ml}$  but not at 1  $\mu\text{g}/\text{ml}$  for treatment of infected L929 cells, whereas 0.5  $\mu\text{g}/\text{ml}$  was effective for infected macrophages. Thus drugs or combinations of drugs more potent than primaquine may be needed to treat certain types of infected cells. Primaquine is concentrated in skeletal muscle, heart, liver, lung, and brain (Andrews et al., 1986); and primaquine thus may be useful for treatment singly or in combination if these are the sites of infection in the chronic stage.

#### ACKNOWLEDGMENT

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## CORRIGENDUM . . .

We regret that the following errors occurred in the paper by Cathy A. Beck and Donald J. Forrester in *The Journal of Parasitology* 74: 628–637, 1988:

- A. Page 628: In the author line, "Cathy Beck" should read "Cathy A. Beck."
- B. Page 629: The first sentence in the right-hand col-

umn should read "Data on intensity were tested for associations with host factors using the Mann-Whitney *U*-test and the Kruskal-Wallis test (Sokal and Rohlf, 1981)."

- C. Page 636. In the reference citation for Dailey et al. (1988), "(in press)" should read "11: 159–163."



# METAZOAN PARASITE COMMUNITY COMPOSITION AND STRUCTURE OF MIGRATING WILSON'S PHALAROPE, *STEGANOPUS TRICOLOR* VIELLOT, 1819 (AVES), FROM EL PASO COUNTY, TEXAS

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**ABSTRACT:** The helminth species constituting the community composition of migrating Wilson's phalarope, *Steganopus tricolor*, from El Paso County, Texas, was neither species rich nor abundant. Of the spring and fall migrants examined ( $n = 100$ ), a total of 828 helminth parasites representing 12 different species was collected. Wilson's phalaropes from this study were also infested with 3 species of mallophagans and 1 species of nasal mite. Despite low species richness and abundance, the helminth community did show community composition and structure.

Based on Shannon's test for diversity, Simpson's index for dominance, interspecific association analyses, dispersion indices, and other tests, helminths were not very diverse or evenly distributed, had little concentration for dominance, showed no significant associations among pairs of helminth species, and were highly aggregated. This pattern was also consistent across the variables of host sex and age, and seasonal migration. Community structure consisted of 4 core and 8 satellite species. Core species were similar for all subsamples and helminth species showed differences with respect to geographical locality and migratory corridors. This study suggests that seasonal migration is the most influential factor in determining community composition and structure in migrating Wilson's phalarope from El Paso County, Texas. A checklist of metazoan parasites reported from Wilson's phalarope from North America is included.

There are 3 species of phalaropes, 2 of which are Holarctic. The third species, Wilson's phalarope, *Steganopus tricolor* (Viellot, 1819), is confined to the Americas.

Research on the metazoan parasites of Wilson's phalarope is limited. *Cyclocoelum ovopunctatum* (Frietas and Ibanez, 1964), *Tanaisia valida* (Ibanez, 1965), and *Echinocotyle ibanenzi* (Rego, 1973) have been reported to infect this bird. Schmidt and Frantz (1972) compared helminths of Wilson's phalarope to its breeding grounds in western Montana to those of spring migrants from northern Colorado en route from southern wintering grounds, and they concluded that most of the parasitic worms were obtained on the summer breeding grounds. According to Schmidt and Frantz, the feeding habits of this bird expose it to many organisms that serve as intermediate hosts or vectors of parasitic helminths.

Wilson's phalarope breeds from British Columbia eastward to Manitoba, locally in Ontario, and south to California, Nevada, Utah, Colorado, Nebraska, and Minnesota. It winters in Chile, Peru, Argentina, and the Falkland Islands (Johnsgard, 1981). This bird is a common migrant in the southwestern United States during

the fall and spring, uncommon in summer, and accidental during the winter season (DiPasquale et al., 1980).

This paper presents information on the metazoan parasite community composition and structure of the Wilson's phalarope, *S. tricolor*. The variables of host sex and age along with seasonal migration also are considered and findings are compared to those reported for other pertinent migratory shorebird species from the southwest.

## MATERIALS AND METHODS

One hundred Wilson's phalaropes, *Steganopus tricolor*, from El Paso County, Texas, were examined for metazoan parasites. Based on records from the El Paso Trans-Pecos Audubon Society, birds collected from May through July were considered spring migrants, and those from August through October were considered fall migrants. Birds were placed in individual plastic bags on ice and those not examined within 6 hr were frozen for later necropsy. Plumage and gonads were used to determine sex, and juveniles were determined by the presence of the bursa of Fabricius.

Acanthocephalans, cestodes, and trematodes were fixed in alcohol-formalin-acetic acid (AFA) and stained in either Semichon's acid carmine or Ehrlich's hematoxylin prior to mounting in Canada balsam or Permount. Ectoparasites were fixed in 70% ethanol, cleared in 10% potassium hydroxide (KOH), and mounted in Lipshaw's mounting medium. Voucher specimens were deposited in the U.S. National Parasite Collection, Beltsville, Maryland 20705 (79306-79320).

Software packages Ecological Analyses-PC, Vol. 1

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and 2, Oakleaf Systems, P.O. Box 472, Decorah, Iowa 52101, and Biostat I and II, A Multivariate Statistical Toolbox, Sigma Soft, Placentia, California 92670, were used, when noted, to assist in analysis. Significance was assumed at  $P \leq 0.05$  unless otherwise noted. Abundance data were used for most of the analyses and terminology follows the definitions of Margolis et al. (1982). Abbreviations in Table IV and Figure 1 are: AC, *Anomotaenia clavigera*; AG, *Anomotaenia gallinagilis*; AD, *Aploparaksis diagonalis*; CS, *Cloacitrema* sp.; CC, *Corynosoma constrictum*; CO, *Cyclocoelum obscurum*; HB, *Hymenolepis brachycephala*; LI, *Leucochloridium insigne*; NA, *Notocotylus attenuatus*; PV, *Plagiorchis vitellatus*; PS, *Plagiorchis* sp.; and TF, *Tanaisia fedtschenkoi*.

Shannon's test for diversity and mean evenness was used to characterize the community in terms of diversity ( $H$ ) and mean evenness ( $J$ ) with confidence intervals calculated for the variables (Zar, 1984). The closer  $J$  values were to 1.00, the more evenly the species of parasites were distributed.

Simpson's index for concentration for dominance among species of helminths was calculated according to Stone and Pence (1978). Concentration for dominance was assumed when  $C \geq 0.25$ .

Interspecific associations between all possible species pairings were computed to determine whether abundance varied in concert, oppositely, or independently. The  $\chi^2$ -statistic with Cole's coefficient was used (interspecific association analysis, Ecological Analysis-PC). The variance/mean ratio for a species of helminth was used to indicate whether its distribution was significantly contagious (clumped, aggregated) (Dispersion Indices, Ecological Analysis Vol. 2-PC, Oakleaf Systems). The degree of overdispersion was obtained by calculating the negative binomial parameter  $k$ , which is an inverse measure of the degree of overdispersion (Cox, 1985).

MANOVA was used to examine the main and interactive effects of the variables sex, age, and seasonal migration on the distribution of the total helminth fauna and on the 6 common species of helminths individually (AOV 2-3, Biostat I).

Classification of importance values ( $I_2$  method) were used to arrange species of helminths in terms of their importance in the parasite community using the method of Pence and Eason (1980). Importance values and community structure were calculated for the common snipe, *Capella gallinago*, from southwestern Texas from data presented by Leyva et al. (1980).  $I_2$  values from Wilson's phalarope were used, in part, to help interpret and compare to clustering results.

Abundance values were used to perform hierarchical clustering on 12 helminth species. The data were log transformed prior to clustering, the algorithm used was group average, and the measure to generate the similarity matrix was percent similarity (SIMDK and CLUST.DEK, Biostat II). Meaningful clusters were selected according to the helminth "core and satellite" species concept of Hanski (1982) and utilized for parasites by Bush and Holmes (1986a, 1986b).

The log-likelihood  $G$ -test was used to test for significant differences in prevalence and the Mann-Whitney  $U$ -test was used to test for significant differences in mean intensities and abundances among the vari-

ables of sex, age, and seasonal migration for individual species of helminths and for all helminth species combined (Zar, 1984). This was used, in part, to compare to the results of MANOVA.

Helminth similarities among species of birds and localities were examined by the use of Sorenson's index of similarity (Stone and Pence, 1978) and the overlap index (Neraasen and Holmes, 1975). The helminth fauna was compared to data from western Montana and northern Colorado reported by Schmidt and Frantz (1972).

## RESULTS

### General comments, helminth community

A total of 828 helminths and 513 ectoparasites was collected from Wilson's phalaropes from El Paso County, Texas ( $n = 100$ ). Sixty-two percent of the birds harbored at least 1 of 12 species of helminth, with a range of 1–4 species and a mean abundance of 8.3. Eighty-four percent were infested with at least 1–4 species of ectoparasites and a mean abundance of 5.1.

The majority of the helminth species (80%) was collected by  $n = 40$ ; no additional species were recorded after  $n = 72$ . All species of ectoparasites were collected by  $n = 9$ .

Among the helminths, the cestodes were highest in prevalence (46%), followed by the trematodes (35%) and acanthocephalans (1%). No nematodes were collected. The most abundant helminths in the total sample were the cestode *Hymenolepis brachycephala* (2.6) and the trematode *Plagiorchis vitellatus* (2.3) (Table I). These 2 species were consistently the most abundant helminths in fall, spring, male, female, adult, and juvenile subsamples. *Quadriceps fimbriatus* was the most abundant ectoparasite (3.2), followed by *Actornithophilus umbrinus* (1.3) and *Saemundssonina tricolor* (0.2) and this, too, was a consistent pattern for all subsamples. The only acarine species collected, the nasal mite *Rhinyssus himantopus*, was low in abundance (0.6), but its mean intensity (10.2) was highest among all ectoparasites (Table I). These were collected from adults only and all but one infected host were spring migrants. Eight species of helminths and the nasal mite *R. himantopus* were established as new host records and are indicated by an asterisk (Table I).

### Community structure in the total sample

The helminth fauna was not very diverse ( $H' = 0.24 \pm 0.09$ ), nor was it evenly distributed ( $J' = 0.75 \pm 0.07$ ). There was little concentration for dominance (0.18) and there were no signifi-



TABLE I. *Helminth fauna of Wilson's phalarope, Steganopus tricolor, from El Paso County, Texas (n = 100).*

Helminth species	Prevalence (%)	Variables*			Intensity			Abundance		
		Sex	Age	Season	$\bar{x}$	SE	Range	$\bar{x}$	SE	Total individuals
Cestoides (4)										
<i>Anomotaenia clavigera</i>	13	B	B	B	9.8	2.9	2–36	1.3	0.5	127
† <i>Anomotaenia gallinagilis</i>	11	B	B	B	10.4	3.8	1–46	1.1	0.5	115
† <i>Aploparaksis diagonalis</i>	5	B	B	B	5.0	1.6	2–11	0.2	0.1	25
<i>Hymenolepis brachycephala</i>	23	B	B	B	11.3	3.2	1–61	2.6	0.9	259
Trematoda (7)										
† <i>Cloacitrema</i> sp.	1	F	A	S	1.0	0.0	—	0.0	0.0	2
<i>Cyclocoelum obscurum</i>	1	M	A	S	1.0	0.0	—	0.0	0.0	6
† <i>Leucochloridium insigne</i>	3	B	B	B	8.7	6.7	1–22	0.3	0.2	26
† <i>Notocotylus attenuatus</i>	5	B	B	B	1.8	0.4	1–3	0.1	0.0	9
<i>Plagiorchis vitellatus</i>	26	B	B	B	8.8	2.1	1–43	2.3	0.6	230
† <i>Plagiorchis</i> sp.	3	F	A	S	1.7	0.7	1–3	0.0	0.0	5
† <i>Tanaisia fedtschenkoi</i>	1	M	A	F	1.0	0.0	—	0.2	0.2	22
Acanthocephala (1)										
† <i>Corynosoma constrictum</i>	1	M	A	S	1.1	0.0	—	0.0	0.0	2
Mallophaga (3)										
<i>Actornithophilus umbrinus</i>	35	B	B	B	3.6	0.4	1–11	1.3	0.2	126
<i>Quadraceps fimbriatus</i>	74	B	B	B	4.1	0.4	1–17	3.2	0.3	306
<i>Saemundsonia tricolor</i>	11	B	B	B	1.8	0.2	1–2	0.2	0.1	20
Acarina (1)										
† <i>Rhinonyssus himantopus</i>	6	B	A	B	10.2	1.9	5–17	0.6	0.3	61

\* S = sex (M = male, F = female); A = age (A = adult, J = juvenile); S = seasonal migrant (S = spring, F = fall); B = both, for each variable.

† New host record.

cant interspecific associations (Table II). For each of the 6 common species of helminths, using the variance/mean ratio test, a significantly clumped pattern of distribution was observed for all subsamples (Table III). The negative binomial parameter,  $k$ , was low in all 6 species, indicating that the degree of helminth aggregation was high and the distributions were overdispersed (Table III).

The results from the MANOVA test indicated that for the variables of host sex and age and for seasonal migration, only seasonal migration was significant for the helminth population as a whole (Table IV). Sex, age, and seasonal migration were significant main effects for *Anomotaenia gallinagilis*, *Anomotaenia clavigera*, and *P. vitellatus*,

respectively (Table IV). There were no significant interactive effects among these variables for the total helminth fauna nor for the 6 common helminth species (Table IV).

According to the  $I_2$  method, there were 4 dominant, 3 codominant, 5 successful, and no unsuccessful immigrant helminth species (Table V). A dendrogram generated from clustering revealed 4 core and 8 satellite species. Core species were identical to the dominant species of the  $I_2$  method and 2-4 of these core species were consistently dominant across the various subsamples. The 4 core species clustered together at 65% similarity (Fig. 1). All core species from all subsamples were mature. The 8 satellite species independently joined the cluster of dominant

TABLE II. *Helminth comparisons for subsamples, Wilson's phalarope, Steganopus tricolor, from El Paso County, Texas.*

Statistical test	Male/female		Adult/juvenile		Spring/fall		Total (n = 100)
	(n = 52)	(n = 48)	(n = 77)	(n = 23)	(n = 55)	(n = 45)	
Shannon's index diversity ( $H$ -test)	0.26 $\pm$ 0.10	0.22 $\pm$ 0.14	0.27 $\pm$ 0.09	0.13 $\pm$ 0.10	0.13 $\pm$ 0.08	0.33 $\pm$ 0.12	0.24 $\pm$ 0.09
Evenness ( $J$ -test)	0.70 $\pm$ 0.11	0.77 $\pm$ 0.15	0.78 $\pm$ 0.08	0.51 $\pm$ 0.02	0.69 $\pm$ 0.20	0.74 $\pm$ 0.03	0.75 $\pm$ 0.07
Simpson's index dominance ( $C$ -test)	0.21	0.19	0.19	0.21	0.18	0.22	0.18
Sorenson's index similarity		73.7		73.7		73.7	—
Overlap index		58.3		58.3		58.3	—

TABLE III. Distribution and degree of aggregation for the common helminth species in Wilson's phalarope, *Steganopus tricolor*, from El Paso County, Texas ( $n = 100$ ).

Helminth species	$S^2/\bar{x}$ ratio	Distribution	$k$
<i>Anomotaenia clavigera</i>	1:4.0*	C†	0.09‡
<i>Anomotaenia gallinagilis</i>	1:4.7*	C	0.08‡
<i>Aploparaksis diagonalis</i>	1:7.0*	C	0.03‡
<i>Hymenolepis brachycephala</i>	1:5.4*	C	0.16‡
<i>Notocotylus attenuatus</i>	1:2.0*	C	0.05‡
<i>Plagiorchis vitellatus</i>	1:1.8*	C	0.51‡

\* Variance significantly larger than mean.

† C = contagious.

‡ Significantly aggregated.

species at increasing distances ranging from 53 to 61% similarity.

#### Sex, age, and seasonal migration subsample comparisons

Ten helminth species were collected from males, 9 from females, all 12 from adults, 7 from juveniles, 11 from spring migrants, and 8 from fall migrants. Seven of the species were common to all subsamples (Table I). The helminth fauna between sexes (male and female), ages (adult and juvenile), and seasonal migrants (spring and fall) showed little diversity, an unevenly distributed helminth fauna, very little concentration for dominance, no significant interspecific associations, and an overdispersed helminth population (Table II). Total prevalences, mean intensities, and abundances were significantly higher for fall-migrating Wilson's phalaropes, but there was no significant difference between adults and juveniles and between males and females.

The trematode *P. vitellatus* was significantly more prevalent and abundant in the fall. The

cestode *A. clavigera* was significantly more intense in the fall, and the cestode *A. gallinagilis* was significantly more intense in fall adult and in juvenile birds.

Sorenson's and species overlap indices for helminths for each of the 3 variables of sex, age, and seasonal migration were the same, and each showed high similarity (73.7%) and species overlap (58.3%) (Table II). Although the similarity of the helminth faunas of migrating spring and fall birds from El Paso, Texas, was high (73.7%), there was very little similarity and species overlap between Texas and northern Colorado spring migrants (13% similarity and 6% species overlap).

#### Helminth faunal comparisons with other shorebird species

Helminth species of Wilson's phalaropes did not show much similarity to those reported for the common snipe, *Capella gallinago* (13%) (Leyva et al., 1980); American avocet, *Recurvirostra americana* (0%) (Garcia and Canaris, 1987); and black-necked stilt, *Himantopus mexicanus* (5%) (Hinojos and Canaris, 1988), although many helminths from these hosts were collected from birds from the same localities as Wilson's phalarope. For those birds that showed some similarities to the Wilson's phalarope, species overlap was very low, ranging from 3 to 7%.

A checklist of the metazoan parasites reported from Wilson's phalarope from the Americas is included in Table VI.

#### Geographical locality

The helminths revealed similarities and differences with respect to locality. Helminth species

TABLE IV. F values generated by MANOVA for main and interactive effects of seasonal migration, host sex, and host age factors across the 100-sample data set of abundances for 6 common species of helminths from Wilson's phalarope, *Steganopus tricolor*, from El Paso County, Texas.

Factor	Total helminth population	MANOVA					
		Helminth species*					
		AC	AG	AD	HB	NA	PV
Season	10.89†	0.11	3.74	2.80	0.69	0.29	12.78†
Sex	0.06	1.73	6.12†	1.02	0.89	0.30	0.14
Age	2.45	4.39†	2.89	0.33	1.28	0.25	0.20
Season-sex	0.88	4.15	0.49	0.58	4.49	0.90	0.12
Season-age	5.13	13.30	2.58	0.67	4.17	1.90	0.95
Sex-age	0.61	1.97	5.20	0.00	0.51	0.11	2.23
Season-sex-age	93.92	96.31	96.25	92.08	102.53	92.20	92.61

\* See Materials and Methods for helminth species abbreviations.

† Significant at  $P < 0.01$ .



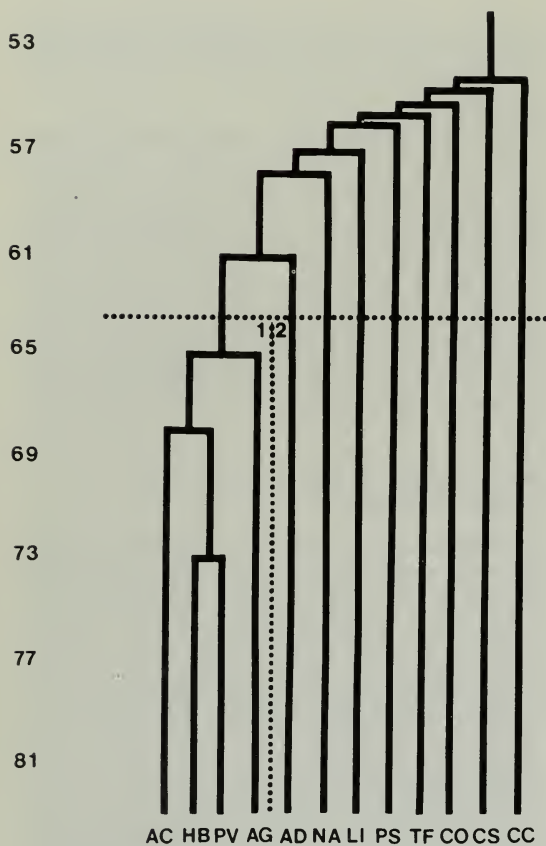


FIGURE 1. Dendrogram of helminth core (1) and satellite (2) species using percent similarity (n = 100).

from El Paso, Texas (n = 100), were somewhat similar to Montana birds (n = 50) (38% similarity and 24% species overlap) and not very similar to northern Colorado birds (n = 45) (12% similarity and 7% species overlap). The closest relationship occurred between western Montana and northern Colorado (46% similarity and 30% species overlap).

The cestode *H. brachycephala* was ubiquitous to all regions (Colorado, Montana, and Texas), all seasons (spring, summer, and fall), and was a core species in all of our subsamples. Three species of nematodes were reported from birds collected in Colorado and Montana (Schmidt and Frantz, 1972), but none was observed in our study.

DISCUSSION

Helminth community composition and structure

**Helminth fauna:** Of the 4 charadriids examined from this area (Wilson's phalarope, *Steganopus tricolor*; common snipe, *Capella gallinago*; American avocet, *Recurvirostra americana*; and

TABLE V. Classification and importance values ( $I_2$ ) of helminths, Wilson's phalarope, *Steganopus tricolor*, El Paso County, Texas (n = 100).

Helminths	$I_2$ values
Dominant species	
* <i>Hymenolepis brachycephala</i>	2.59
* <i>Plagiorchis vitellatus</i>	2.29
* <i>Anomotaenia clavigera</i>	1.26
* <i>Anomotaenia gallinagilis</i>	1.15
Codominant species	
† <i>Leucocloridium insigne</i>	0.26
† <i>Aploparaksis diagonalis</i>	0.25
† <i>Tanaisia fedtschenkoi</i>	0.22
Successful immigrants	
† <i>Notocotylus attenuatus</i>	0.09
† <i>Cyclocoelum obscurum</i>	0.06
† <i>Plagiorchis</i> sp.	0.05
† <i>Corynosoma constrictum</i>	0.02
† <i>Cloacitrema</i> sp.	0.02
Unsuccessful immigrants: none	

\* Core species.  
† Satellite species.

black-necked stilt, *Himantopus mexicanus*), Wilson's phalarope had the lowest helminth species richness (12, compared to 14, 19, and 19) and the lowest mean abundance (8.3, compared to 14.9, 126.8, and 162, respectively) (Leyva et al., 1980; Garcia and Canaris, 1987; Hinojos and Canaris, 1988). Wilson's phalarope and the common snipe are transitory migrants. The American avocet and black-necked stilt may linger during migration, but both also nest in this locality. Greater species richness and mean abundances in the American avocet and black-necked stilt may be related to the greater opportunity for these birds to establish and partake from infective pools of helminths in this locality compared to the 2 transitory species.

**Diversity, evenness, and dominance:** Wilson's phalarope was similar to the American avocet and black-necked stilt in that the helminths were not very diverse, were not very evenly distributed, and showed little concentration for dominance. Diversity and evenness values were not available for the common snipe. The abundance of 2 species of helminths for the common snipe made the concentration for dominance higher (0.33) than the quite low figures recorded for the Wilson's phalarope (0.18), American avocet (0.15), and black-necked stilt (0.09) (Leyva et al., 1980; Garcia and Canaris, 1987; Hinojos and Canaris, 1988). From this, and ongoing studies, we have observed that low diversity, unevenness, and low concentration for dominance is char-

TABLE VI. Checklist of helminth parasites of Wilson's phalarope, *Steganopus tricolor*, from North America.

Taxon	Locality	Citation
Cestoidea (7)		
<i>Anomotaenia clavigera</i>	MT, TX	Schmidt and Frantz, 1972; this study
<i>Anomotaenia gallinagilis</i>	TX	This study
<i>Aploparaksis diagonalis</i>	TX	This study
<i>Hymenolepis brachycephala</i>	CO, MT, TX	Schmidt and Frantz, 1972
<i>Hymenolepis calumnacantha</i>	MT	Schmidt and Frantz, 1972
<i>Hymenolepis capellae</i>	CO	Schmidt and Frantz, 1972
<i>Echinocotyle ibanenzi</i>	Peru	Rego, 1973
Trematoda (10)		
<i>Cloacitrema</i> sp.	TX	This study
<i>Cyclocoelum obscurum</i>	MT, TX	Schmidt and Frantz, 1972
<i>Cyclocoelum ovapunctatum</i>	Peru	Frietas and Ibanez, 1964
<i>Leucochloridium insigne</i>	TX	This study
<i>Notocotylus attenuatus</i>	TX	This study
<i>Notocotylus</i> sp.	MT	Schmidt and Frantz, 1972
<i>Plagiorchis vitellatus</i>	MT, TX	Schmidt and Frantz, 1972; this study
<i>Plagiorchis</i> sp.	TX	This study
<i>Tanaisia fedtschenkoi</i>	TX	This study
<i>Tanaisia valida</i>	Peru	Ibanez, 1965
Nematoda (3)		
<i>Echinuria skrjabinensis</i>	CO, MT	Schmidt and Frantz, 1972
<i>Stellocaronema skrjabini</i>	MT	Schmidt and Frantz, 1972
<i>Tetrameres dubia</i>	CO, MT	Schmidt and Frantz, 1972
Acanthocephala (1)		
<i>Corynosoma constrictum</i>	TX	This study

acteristic for helminths of charadriids from this locality and is probably related to the overdispersed distribution of helminths.

**Distribution:** The contagious and overdispersed (low  $k$  values) distributions of the common species of helminths in Wilson's phalarope were consistent for the total sample and all subsamples. Contagious distributions with low  $k$  values were also reported for the common helminth species in the American avocet and black-necked stilt from this same locality, except for a uniform distribution for several species of large tapeworms infecting the latter 2 species of birds (Garcia and Canaris, 1987; Hinojos and Canaris, 1988). Wallace and Pence (1986) also reported a consistently overdispersed distribution for the common species of helminths among the subsamples for the blue-winged teal, *Anas discors*, from the Texas panhandle. Overdispersed populations of helminths appear to be a common phenomenon (Anderson, 1982; Pence and Windberg, 1984; Wallace and Pence, 1986). Anderson (1982) attributed overdispersion to heterogeneity within the host population (genetic variability, host susceptibility, and spatial location of the parasites). Wallace and Pence (1986) suggested that additional factors such as behavioral and social traits, individual feeding characteristics,

and chance distribution of infective stages also contributed to individual host variability in determining the magnitude of helminth infections in specific host individuals. These factors appear to be applicable in explaining overdispersion and may also be contributing to low diversity and evenness in helminths infecting Wilson's phalarope.

**Core and satellite species:** Both clustering and the  $I_2$  method were useful in quantitatively assessing the relative importance of helminth species within an infracommunity. They showed that Wilson's phalarope did harbor a primary community (core/dominant species) of a few relatively abundant (88% of all helminths), essentially constant helminth species and a secondary community (satellite/nondominant species) that were more variable and less abundant across subsamples. Some associations and correlations among core species were reported for the black-necked stilt from this locality, where they breed (Hinojos and Canaris, 1988), and between the 2 core species from the long-billed curlew, *Numenius americanus*, collected on the breeding grounds in Alberta, Canada (Goater and Bush, 1987). Associations among the core species were not demonstrated for Wilson's phalarope. This may have been influenced by the relatively low



abundances observed for each species of helminth. Whether associations, negative or positive, can be demonstrated among helminths for Wilson's phalarope from the breeding and wintering grounds needs to be examined.

**Core species:** Both of the transitory migrants, Wilson's phalarope and the common snipe, from our locality had fewer core species than the 2 breeding species, the American avocet and black-necked stilt, from the same locality: 4, 3, 8, and 10, respectively (Leyva et al., 1980; Garcia and Canaris, 1987; Hinojos and Canaris, 1988). Goater and Bush (1987) reported only 2 core species and low species richness (9) for the long-billed curlew collected on the breeding grounds in Alberta, Canada. It does appear that we can expect differing numbers of core species and the lowest numbers may not necessarily be observed in charadriids that are in migration. There is also some indication from Wilson's phalarope and the common snipe studies (Leyva et al., 1980) that one can expect about the same core species (4 of 4, Wilson's phalarope; 2 of 3, common snipe) to be found in both fall and spring migrants. All the core species in both spring and fall birds were mature, but because we did not know how rapidly these helminths matured, we could not determine if the core species were being sustained by reinfection or by the longevity of the parasite, or both.

**Satellite species:** Satellite species do appear to be more eclectic between fall and spring for both Wilson's phalarope and common snipe (both seasons 3, 2; fall 1, 5; spring 4, 5, respectively). Some variability was also evident between fall and spring migrants for the less common helminth species in the blue-winged teal (Wallace and Pence, 1986).

**Sex, age, and seasonal migration:** Seasonal migration seems to be the most important factor influencing the numbers of helminths, with total abundance higher in fall ( $\bar{x} = 12.6$ ) than in spring migrants ( $\bar{x} = 4.8$ ). It was the only significant main effect and there were no interactive effects among the variables of sex, age, and seasonal migration. Wallace and Pence (1986) observed in the blue-winged teal that the main and interactive effects of host season (which compared fall and spring migrants) and age accounted for the variance for many of the helminth species and that 4 species varied across the main effect of season only. Some of the more important intervening variables implicit in seasonal migration that may be influencing changing abundances are

variable longevity for species of parasites, availability of infective parasite stages, and perhaps migratory stress. Immune competency, achieved through age, was postulated to be a factor in changing abundances in the blue-winged teal from the Texas panhandle (Wallace and Pence, 1986). Because age was not a significant main or interactive effect, immune competency may not be as significant a factor in altering abundances in Wilson's phalarope as was observed in the blue-winged teal.

#### Helminth sharing among hosts

Suitable habitat for charadriids in El Paso County, Texas, is very limited, and all 4 species of birds (Wilson's phalarope, common snipe, American avocet, and the black-necked stilt) examined thus far from this area utilize the same general habitats. Only 2 species of helminths collected from Wilson's phalarope were recovered from any of the other 3 species of birds collected from this same locality. None of the helminths was a core species, and 1 (*Tanaisia fedtschenkoi*) of the 2 shared with the common snipe and the black-necked stilt is a ubiquitous parasite among aquatic birds.

Garcia and Canaris (1987) also reported only 3 of 38 species of helminths shared between the American avocet and the black-necked stilt collected from the same locality in El Paso County; these birds are closely related, nest in this locality, and migrate through this area. Parasite specificity, feeding habits, and availability of intermediate hosts in this locality may be some of the important factors in explaining the very low similarities among the host species.

#### Geographical locality

The helminth ecology of Wilson's phalarope revealed both similarities and differences with respect to geographical localities. There were differences among the helminth faunas of birds from Texas, Colorado, and Montana. Montana and Colorado birds were most similar, which probably reflects the similar environments of these nearby geographical populations. Thul et al. (1985), in their investigation of wood ducks, *Aix sponsa* at 24 localities on the Atlantic coast, found that the greatest similarities among the helminth faunas tended to occur among geographical neighbors.

Birds from Montana and Colorado harbored nematodes, none of which was collected from birds from El Paso County, Texas. This absence

may be related to seasonal dietary differences, the absence of or unavailability of infective larval stages, and absence of intermediate hosts in our area. The one acanthocephalan, *Corynosoma constrictum*, in a spring migrant can be considered accidental. This species is usually a parasite of anseriforms and gruiforms (McDonald, 1969) and has been reported from the green-winged teal, *Anas crecca*, on the wintering grounds in Texas (Canaris et al., 1981); the blue-winged teal (Wallace and Pence, 1986); and from the non-migratory Mexican duck, *Anas platyrhynchos diazi*, from southwestern Texas and Mexico (Farias and Canaris, 1986).

Helminth species from Texas and Colorado were less similar than helminth species from Texas and Montana. An explanation may be that birds from western Montana and southwestern Texas utilize the same migratory corridor, mostly west of the Rocky Mountains, but that birds from northern Colorado, east of the Rocky Mountains, use a different corridor. There are, from our and other studies, indications of community differences among helminths in the same species of bird using different migratory corridors (Gaines et al., 1984; Thul et al., 1985; Wallace and Pence, 1986; Garcia and Canaris, 1987).

Whether these and other community patterns along with the influences of sex, age, migration, and season will be the same or differ on the breeding and wintering grounds for Wilson's phalarope is presently unknown and needs to be examined.

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## GM-CSF PRODUCED BY RECOMBINANT VACCINIA VIRUS OR IN GM-CSF TRANSGENIC MICE HAS NO EFFECT *IN VIVO* ON MURINE CUTANEOUS LEISHMANIASIS

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**ABSTRACT:** The hemopoietic growth and differentiation regulators, granulocyte-macrophage colony-stimulating factor (GM-CSF) and the multipotential stimulating factor (multi-CSF) have been shown to have major effects on the effector function of mature macrophages. In this study we have examined the effect of recombinant GM-CSF and multi-CSF expressed transiently from recombinant vaccinia virus, or constitutively in GM-CSF transgenic mice on the development of cutaneous leishmaniasis, caused by *Leishmania major* in genetically susceptible or resistant mice. We observed no effect on the development of lesions when GM-CSF or multi-CSF were administered before infection, nor on the healing of lesions when they were administered after appearance of lesions. Although only some of the GM-CSF transgenic mice or their normal littermates developed lesions after infection with *L. major*, there was no difference between the groups in the rate of lesion development or in the size of lesions.

The hemopoietic growth and differentiation regulator, granulocyte-macrophage colony-stimulating factor (GM-CSF) has been shown to have a potent effect on the effector function of mature phagocytes (Metcalf, 1986; Clark and Kamen, 1987). This makes GM-CSF attractive as a candidate drug to control intramacrophage infections, in which the invading organisms are destroyed intracellularly by the activated host cell. Cutaneous leishmaniasis is an ideal model system to determine the therapeutic usefulness of this hemopoietic growth and differentiation regulator because *Leishmania major* is an obligatory intracellular parasite in macrophages. In mouse strains that are resistant to disease, T cell-mediated immune mechanisms are responsible for the spontaneous healing process. Cytokines secreted by these T cells have been implicated in the macrophage activation leading to intracellular parasite killing. Our earlier studies suggested that GM-CSF, in addition to  $\gamma$ -interferon (Titus et al., 1984; Saddick et al., 1986) also played a role in the *in vitro* killing of *L. major* by murine macrophages (Handman and Burgess, 1979).

In this study we examine the effect of genetically engineered GM-CSF expressed transiently from recombinant vaccinia virus, or constitutively in GM-CSF transgenic mice on the development of cutaneous leishmaniasis in mice that are genetically susceptible or resistant to infection with *L. major*.

### MATERIALS AND METHODS

#### Construction of plasmids containing the genes for hemopoietic growth factors

CSF cDNAs were cloned into the vaccinia transfection vector pGS62 at a site adjacent to the 7.5-kDa early gene promoter. This plasmid was derived from pGS20 (Mackett et al., 1984) by deletion of an EcoRI restriction site. The GM-CSF cDNA was a ~770-bp BamHI-EcoRI fragment from a clone designated pGM3.2Δ17 in Gough et al. (1985). It contains the entire GM-CSF coding sequence, starting from the 3'-most of 2 alternative initiation codons. The multi-CSF cDNA was a ~1.35-kb EcoRI fragment containing all coding sequences, and some 5' and 3' untranslated sequences (Gough et al., 1985; Tsuchiya et al., 1986).

#### Production of recombinant vaccinia viruses expressing murine hemopoietic growth factors

Recombinant vaccinia viruses were produced as described by Langford et al. (1986). Thymidine kinase-deficient (tk<sup>-</sup>) viruses were screened for the presence of CSF sequences by hybridization of <sup>32</sup>P-labeled probes to dot blots of extracts from infected cells. The purified viruses were designated vGM-CSF and vmulti-CSF.

#### CSF secretion by cells infected with recombinant viruses

Confluent 50-mm dishes of BSC-1 monkey kidney cells were infected with either vGM-CSF, vmulti-CSF, or a tk<sup>-</sup> control vaccinia virus, at a multiplicity of 1 PFU/cell. After 2 hr at 37 C, the virus inoculum was removed and replaced by 2 ml of serum-free culture medium. After incubation at 37 C for 24 hr, the medium was removed, centrifuged at 12,800 g for 5 min, and treated with  $\beta$ -propiolactone to inactivate virus (LoGrippo, 1960). Supernatants were buffered by addition of 0.05 ml of 2.8% NaHCO<sub>3</sub> and 0.02 ml of 1 M HEPES, pH 7.6, per ml of supernatant, made 0.1% with  $\beta$ -propiolactone, and incubated at 4 C overnight. Finally, the solutions were incubated at 37 C for 3 hr to inactivate residual  $\beta$ -propiolactone.

GM- and multi-CSF levels in the culture media were

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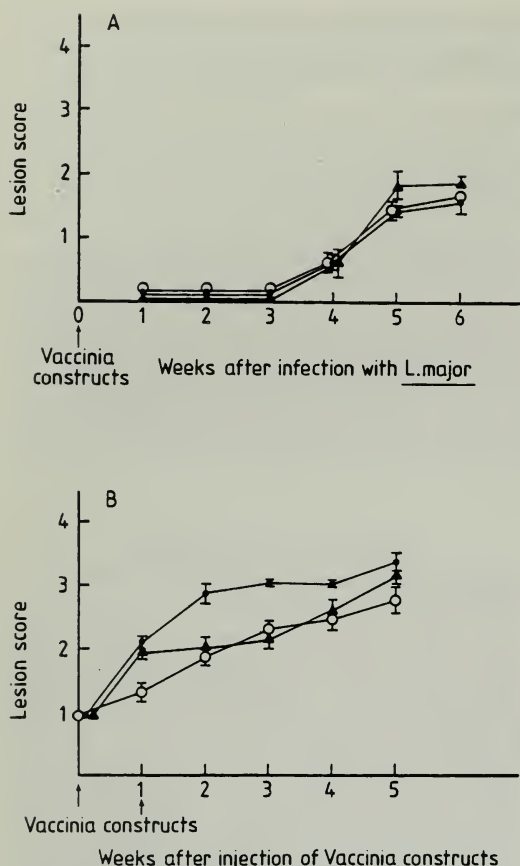


FIGURE 1. Lesion scores in BALB/c.H-2<sup>k</sup> mice injected intraperitoneally with vaccinia virus control (tk<sup>-</sup>) (●—●), vGM-CSF (▲—▲), or vmulti-CSF (○—○) and immediately infected intradermally with *L. major* promastigotes (A), or mice that had been infected with *L. major* on day -20 and then on days 0 and +7 were injected intraperitoneally with the various vaccinia constructs (B).

measured using factor-dependent cell lines as described below.

#### CSF bioassays

Levels of GM-CSF or multi-CSF in serum or conditioned media were assayed using microwell cultures of either FDC-P1 cells (responding to stimulation by either GM-CSF or multi-CSF) or 32D cells (responding only to stimulation by multi-CSF). In the standard assay, 5- $\mu$ l volumes of serial 2-fold dilutions of the test material were added to 10- $\mu$ l volumes of Dulbecco's modified Eagle's medium containing 20% fetal calf serum and either 200 FDC-P1 cells or 200 32D cells in 60-well microtiter trays. After 48 hr of incubation at 37 C in a fully humidified atmosphere of 10% CO<sub>2</sub> in air, cell counts on all wells were performed using an inverted microscope. The dilution resulting in 10-30 viable cells was used as the end point of the titration and was converted into conventional bone marrow CSF

units/ml (50 units/ml is the concentration stimulating half maximal numbers of colonies to develop in agar cultures of 75,000 C57Bl/6 bone marrow cells) by parallel titration of standard preparations of purified native GM-CSF or multi-CSF of known colony-stimulating activity.

#### Parasites

The virulent cloned line V121 was derived from a human isolate of *L. major* by limit dilution cloning and has been extensively characterized (Handman et al., 1983).

Parasites were maintained *in vitro* in Schneider's *Drosophila* medium (GIBCO) supplemented with 10% fetal calf serum. They were used in their stationary phase of growth, usually on day 5 in culture. Parasites were washed in phosphate-buffered saline (PBS), pH 7.3, resuspended at a concentration of  $6 \times 10^6$ /ml, and a volume of 100  $\mu$ l was injected intradermally at the base of the animal's tail. Development of lesions was monitored weekly and lesion size was scored as previously described (Mitchell and Handman, 1983).

#### Mice

The mice used for infection with the vaccinia viruses described above were BALB/c H-2<sup>k</sup> females 7-10 wk old. Mice of BALB/c genotype are relatively susceptible to infection with *L. major*, but among the H-2 congenic mice on this background there is a gradation in susceptibility: BALB/c > BALB/c H-2<sup>b</sup> > BALB/c H-2<sup>k</sup>. Thus, BALB/c H-2<sup>k</sup> may recover from disease, or disease may progress more slowly.

The transgenic mice carrying the continuously expressed GM-CSF gene have been described in detail elsewhere (Lang et al., 1987). These mice and their nontransgenic littermates were SJL  $\times$  C57Bl/6 F<sub>2</sub>, which are relatively resistant to infection with *L. major* (Handman et al., 1979).

#### RESULTS

In the first experiment, groups of 5 mice were injected intraperitoneally with  $10^8$  PFU of tk<sup>-</sup> vaccinia virus, vGM-CSF, or vmulti-CSF, or were left untreated. They were simultaneously infected intradermally with *L. major* promastigotes and were monitored weekly for development of lesions. There was no significant difference between the groups in the rate of lesion development or in the size of the lesions (Fig. 1A). Blood samples were taken at 3-day intervals and the sera from each group were pooled. An aliquot of each bleed was used to measure viremia. Serial dilutions of each aliquot were absorbed onto confluent monolayers of BSC-1 cells, and the cells were incubated for 7 days at 37 C. The cultures were checked daily for signs of viral infection, and after 7 days, they were stained with crystal violet to detect viral plaques. There was no virus detectable in any of the serum samples assayed. A second aliquot of each pool was treated with

$\beta$ -propiolactone, as described above, and assayed for CSF activity, or for anti-vaccinia antibodies.

Those mice infected with the tk<sup>-</sup> virus showed no increase of CSF levels in their sera above that measured in the prebleed. However, mice infected with vGM-CSF or vmulti-CSF showed a dramatic, though transient, elevation in circulating levels of the expected CSF (Fig. 2A). The burst of CSF production was very rapid, with levels returning to the control value by day 6. To measure more accurately the kinetics of CSF appearance, i.e., the lag time and time of peak production, it would be necessary to assay sera at more frequent intervals than we used in this experiment. The limited duration of CSF production was undoubtedly determined, at least in part, by the immune response mounted against the vaccinia virus. Anti-vaccinia antibodies were easily detected in mouse serum 3 days after infection (Fig. 2B) and reached plateau levels after 10 days. Nevertheless, for a short period, infection with these CSF-secreting recombinant viruses resulted in a marked increase in serum CSF levels. *Leishmania*-induced lesions, however, appeared only about 3 wk after infection, when serum levels of these regulators had dropped to their normal (undetectable) levels (data not shown).

In a second experiment, mice were infected with *L. major* promastigotes, and when all mice had developed small swellings at the site of infection, they were divided into groups and injected with the vGM-CSF, vmulti-CSF, or tk<sup>-</sup> vaccinia virus. A second equivalent vaccinia injection was given 1 wk later. The timing of infection and multiple dose were designed to elevate serum CSF levels coincident with the time of active infection of macrophages by *L. major* parasites. *Leishmania major* infection on its own did not increase the level of CSF detectable in serum above that of uninfected controls. Mice were tested 2 wk after infection and at 3-wk intervals for 4 mo (data not shown). Figure 1B shows that no significant difference in lesion development was observed between mice receiving vGM-CSF, vmulti-CSF, or the tk<sup>-</sup> control virus.

Thus, a third type of experiment was done using transgenic mice that express the GM-CSF transgene continuously. These mice constitutively exhibit levels of GM-CSF in the serum that approach 3,000 U/ml (normal levels  $90 \pm 90$  U/ml) (Lang et al., 1987). A total of 9 transgenes and 12 normal littermates were infected

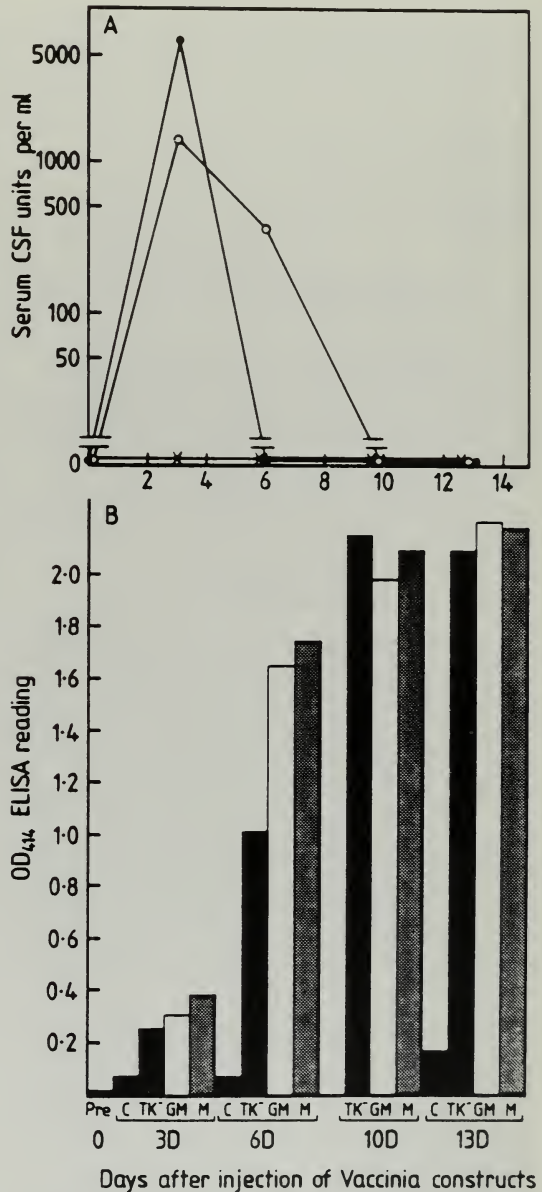


FIGURE 2A. Time course of appearance of vGM-CSF or vmulti-CSF in the circulation of mice injected with vaccinia control (tk<sup>-</sup>) (x—x), vGM-CSF (●—●), or vmulti-CSF (○—○) as described in Figure 1A. B. Time course of appearance of anti-vaccinia antibodies in the same serum samples tested in Figure 2A including untreated *L. major* infected mice (C) and serum from the experimental mice prior to the injection of vaccinia virus (PRE).

intradermally with *L. major* and lesion development monitored. Only 5 of the control mice and 5 of the transgenic mice developed lesions. Nevertheless, the rate of lesion development or



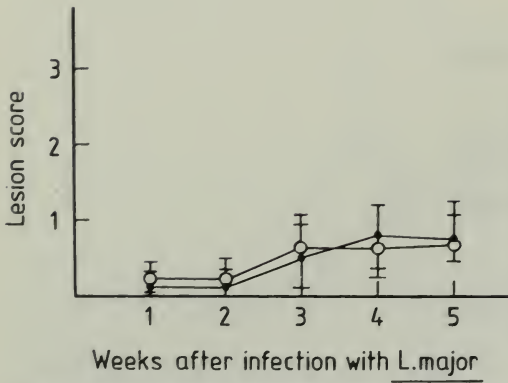


FIGURE 3. Lesion scores in GM-CSF transgenic mice (●—●) and their normal littermates (○—○) after intradermal injection of *L. major* promastigotes.

the size of the lesion was similar in both groups (Fig. 3).

### DISCUSSION

The murine model of cutaneous leishmaniasis represents a useful system for the analysis of cellular parameters involved in susceptibility or resistance to infection with *L. major*. Depending on the genetic background of the mice, the entire spectrum of the clinical manifestations of human disease can be observed. Following intradermal injection of *L. major* promastigotes, resistant mice (CBA/H, C57Bl/6, or C3H/He) develop small lesions that heal spontaneously, whereas susceptible mice (BALB/c and its H-2 congenic lines) develop nonhealing lesions (Handman et al., 1979).

A considerable body of evidence indicates that specific T-cell responses generated during infection play a major role both in the resolution and in the progression of disease (reviewed in Handman, 1986). It has been suggested that in the susceptible animals, T cells fail to produce certain lymphokines, such as  $\gamma$ -interferon, that are required to activate macrophages to kill the intracellular organisms.

GM-CSF has proven to be both a growth stimulator of immature hemopoietic cells and a potentiator of responsiveness of mature phagocytes exposed to infectious organisms (Metcalf, 1986; Clark and Kamen, 1987). Therefore, murine cutaneous leishmaniasis seemed an ideal system to examine the effect of GM-CSF on the survival of an obligatory intracellular parasite of macrophages.

Our earlier studies using relatively pure GM-CSF indicated that peritoneal macrophages could

be activated *in vitro* to accelerate killing of *L. major*. This was subsequently questioned by Titus et al. (1984) who showed that the activation and intracellular killing could be ascribed to  $\gamma$ -interferon alone. However, from studies by Nacy et al. (1985) it is clear that mediators other than  $\gamma$ -interferon also play a role in parasite killing. To clarify this point, we designed a series of *in vitro* experiments in which a recombinant vaccinia virus carrying GM-CSF or multi-CSF genes would deliver these growth mediators into the mouse circulation. We observed no effect on the development of lesions, when GM-CSF or multi-CSF were administered before infection, nor on the healing of lesions, when they were administered after appearance of lesions. However, the production of GM-CSF or multi-CSF was short-lived and detectable for only a few days after injection of the recombinant vaccinia viruses. Leishmaniasis is a chronic disease that may be caused by a single breakthrough parasite (Handman et al., 1983) and the time scale for lesion development or healing is many weeks. So, it is possible that if these mediators do play a role in parasite killing by macrophages, their presence in the circulation or, more importantly, locally, within the lesion, needs to be prolonged. To examine this point, we used transgenic mice that constitutively express the exogenous GM-CSF gene from retroviral regulatory sequences continuously. The level of GM-CSF in the serum of these mice is similar to that detected in serum of mice injected with vGM-CSF, whereas GM-CSF levels are almost undetectable in normal mice.

Only 5 of the 9 transgenic mice and 5 of 12 normal littermates developed lesions when infected with *L. major*, probably because of the genetically resistant background of SJL  $\times$  C57Bl/6 mice. However, there was no difference between the groups in the rate of lesion development, nor in the size of the lesion. Because the transgenic mice usually die within a few weeks of causes unrelated to the *Leishmania* infection (Lang et al., 1987), we could not examine the rate of healing.

The data presented here suggest that GM-CSF does not play a major role in activating macrophages to kill the obligatory intracellular organism *L. major*. A study by Solbach et al. (1987), using injections of recombinant GM-CSF to moderate *Leishmania* infection in genetically susceptible BALB/c mice, showed no effect on half the mice injected, and exacerbation of dis-

ease in the other half. Moreover, new evidence is accumulating that multi-CSF injection of BALB/c mice also results in the development of more severe lesions (Louis et al., 1987). They propose that these regulators exacerbate the development of lesions by increasing the pool of circulating macrophages recruitable to the lesions, thus favouring the multiplication of parasites. These cells cannot be activated due to a genetic defect in production of  $\gamma$ -interferon in this mouse strain. This may also be the case in the GM-CSF transgenic mice that exhibit greatly increased numbers of macrophages. Immature macrophages from the bone marrow, accumulating in the lesion may be just a highly susceptible target cell population for *Leishmania*. We have not observed any exacerbation of disease in either the genetically susceptible BALB/c H-2<sup>k</sup> or the genetically resistant transgenic mice, but rather a lack of effect of GM-CSF on the course of *L. major* infection as measured by lesion development.

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## SEXUAL BIAS IN HOST SELECTION BY PARASITIC MITES OF THE MOSQUITO *ANOPHELES CRUCIANS* (DIPTERA: CULICIDAE)

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**ABSTRACT:** Potential mechanisms leading to higher parasitic water mite abundances on female *Anopheles crucians* were tested in laboratory experiments. Females and males had similar pupal stage durations and so are probably accessible to mites for similar time periods. Significantly more mites attached to female than male pupae in equal-exposure tests, even when females had been reared on a reduced food level that caused them to be smaller than males. Also, female pupae, whether normal or small, were not less capable than males at foiling mite attachment. Thus, the greater mite abundance on female pupae probably reflects mite preference. Despite this preference for female pupae, mites still attach to male pupae. Although mites on female mosquitoes probably return to water more consistently because of mosquito oviposition, attachment to males may not be strongly selected against. The short life span of unattached mite larvae and a potentially low encounter rate between mites and pupae may favor a mite's remaining on a male pupa, as long as the probability of returning to water from a male host is greater than 0.

Female mosquitoes often bear more parasitic water mites of the genus *Arrenurus* than do males in natural populations (McCrae, 1976; Stechmann, 1980; Smith and McIver, 1984a, 1984c). This observation is seemingly a logical consequence of natural selection: these mites, which first attach to mosquito pupae and then transfer to and parasitize emerging adults, can complete their life cycle only if they return to water, a more probable event on female hosts because they oviposit in or near aquatic sites (Mullen, 1974; Stechmann, 1980; Smith and McIver, 1984a). The greater abundance of mites on female mosquitoes, however, does not necessarily imply that mites select females over males, and previous laboratory studies have failed to demonstrate sexual bias in host selection (Smith, 1988). Greater mite abundance on females may be caused simply by a greater temporal overlap between emerging mites and female pupae. For example, *Arrenurus angustilimbatus* Mullen was more abundant on female mosquitoes in field samples (Smith and McIver, 1984b), but showed no sexual preference when exposed to pupae of *Aedes excrucians* (Walker)—and of 3 other species of *Aedes*—in laboratory experiments (Smith and McIver, 1984b). In both *A. excrucians* and *Coquilleltidia perturbans* (Walker), higher infestations on female mosquitoes have been attributed to the closer synchrony between mite and female emergence than between mite and the earlier male emergence (Smith and McIver, 1984a, 1984c).

Temporal overlap of emerging mites and fe-

male hosts could result from natural selection if the host sexes have different emergence periods, as in the univoltine mosquitoes studied by Smith and McIver (1984a, 1984b, 1984c), but not if the host sexes have similar and practically continuous emergence patterns, as in the Florida population of the mosquito *Anopheles crucians* Wiedemann included in the present study. Newly emerged adult *A. crucians* of each sex have been collected during most months near the shore of Lake Alice, Gainesville, Florida, and females have higher abundances of the mites *Arrenurus novimarshallae* Wilson and *Arrenurus pseudotenuicollis* Wilson than do males (Lanciani, 1979b, 1987, and pers. obs.). Thus, in these mosquito-mite associations, a mechanism (or combination of mechanisms) other than temporal overlap is causing higher mite abundances on female hosts.

This study of the *A. crucians*-*A. novimarshallae* association had 2 objectives: first, to measure the duration of the mosquito's pupal stage, for if it is longer in females than in males, female pupae may be vulnerable to mites for longer periods, allowing more mites to attach; and second, to compare the number of mites on pupae of each sex in equal-exposure tests. The claim that greater mite numbers on female pupae reflect true mite preferences was supported by experiments run to discern any influence of pupal body size and mite-avoidance behavior on the number of attached mites.

### MATERIALS AND METHODS

#### Mite species identification

The mite species was identified as *A. novimarshallae*, which is quite similar to *A. pseudotenuicollis* but differs

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in having a distinct rounded hump dorsally on the male cauda (Wilson, 1961, 1986). Both of these mite species have been recovered from the same individuals of *A. crucians* in field samples from Florida (pers. obs.). Previous publications on the *A. crucians*–*A. pseudoten-uicollis* association have mentioned the likely involvement of more than 1 mite species (Lanciani and Boyt, 1977; Lanciani, 1979a, 1979b, 1987).

### Pupal duration

The length of time each sex of *A. crucians* spent in the pupal stage was measured in the laboratory. Gravid females were collected from a woodland site next to Lake Alice in mid-December 1986 and were placed in screen-covered vials containing tap water. The vials were held in the dark at 28 C in covered, moistened containers until eggs were deposited. From 200 to 300 eggs were transferred to each of several white enamel pans containing 500 ml of tap water maintained at 28 C, and approximately 36 hr after oviposition, 0.10 mg of a 2:1 mixture of TetraMin® baby-fish food and brewer's yeast was added to each pan. Two days later larvae were separated into groups of 100, placed into white enamel pans containing 500 ml of tap water, and provided with 0.12 mg of the fish food–brewer's yeast mixture. After 2 more days, larvae were transferred to fresh water with 0.14 mg of food. On each subsequent day, the water was changed and 0.18 mg of food was added until pupation. The larvae were kept in a reach-in constant-environment chamber at 28 C with a light-dark cycle of 12 hr. As larvae approached pupation, they were checked each hour, and pupae found were assumed to have reached that stage at the ½-hr mark. Pupae of known age were then placed in screen-covered vials of tap water and returned to the constant-environment chamber. As pupae approached adult-emergence time, they were checked each hour, and adults found were also assumed to have reached that stage at the ½-hr mark. Thus, the entire pupal period for any individual was estimated with a maximum error of 1 hr. Pupal durations were measured on 29 males and 39 females; the difference between means was evaluated with a 1-tailed *t*-test. The null hypothesis was females and males have the same pupal periods, and the alternate hypothesis was females have longer pupal periods.

### Equal-exposure tests

Pupae of each sex were equally exposed to mites of *A. novimarsallae* in laboratory experiments to test whether mites actively select females over males. A possible confounding feature to testing pupal selection, even given equal exposure to mites, is pupal body size. Because female pupae of *A. crucians* are larger than male pupae grown under the same conditions, they present a greater contact area to mites. Consequently, more mites may encounter female pupae even if no preference for female pupae exists. The influence of pupal body size on mite selection was tested by matching individuals reared on a reduced food level (one-tenth the normal amount) with individuals reared on the standard food level. In the following equal-exposure experiments that were run, "small" indicates those individuals reared on the reduced diet and "normal" indicates those reared on the standard diet: (1) normal males vs. normal females, to test the influence of pupal

sex on mite selection; (2) normal males vs. small females, to help prevent body size from clouding the interpretation of experiment 1; and (3) small males vs. normal females, to help prevent food level from clouding the interpretation of experiment 2. Additional experiments run to help remove body size as a confounding variable were (4) normal vs. small males and (5) normal vs. small females. After the experiments, pupae were dried for 24 hr at 60 C and weighed with a Cahn G-2 electrobalance to verify the expected differences in size.

Each experiment consisted of 8 trials, and each trial included 4 pupae from each of the 2 groups to be contrasted, yielding 64 pupae per experiment. All experimental individuals had entered the pupal stage approximately 24 hr earlier, and those used in the same trial were of the same relative pupal age as judged by the degree of appendage pigmentation of the developing adult. In each trial, the 8 pupae were placed together into 1 of 5 plastic aquaria each holding 1.5 L of water and were removed after exactly 2 hr. These aquaria, which are housed in a constant-environment chamber maintained at 28 C and with a 12-hr light-dark cycle, have contained *A. novimarsallae*, ostracods (providing food for nymphal and adult mites), and a layer of flocculant algae for more than 3 yr. Periodic additions of mites have assured a steady supply of larvae—the parasitic stage of the mite. After exposure to mites, each pupa was collected with a wide-tipped pipette and placed individually in a vial containing a few milliliters of water. Each pupa was then transferred to a water drop on a depression slide, heated briefly over the flame of an alcohol lamp, and inspected with a dissecting microscope. The heating killed the pupa and any attached mites, so mites could be easily dislodged and counted accurately. Mites found in the holding vials after pupal removal were added to the count of attached mites, but such free mites were rarely present.

The Wilcoxon matched-pairs signed-rank test was used to test the difference between the number of mites on the 2 groups of pupae in each experiment. If the pupae of each group harbored equal numbers of attached mites in a trial, that trial was replaced to ensure a total of 8 statistically usable trials per experiment. The null hypothesis tested was that 2 groups of pupae in an experiment have the same number of mites. The alternate hypothesis in each experiment specified a direction for the difference in number of mites, so 1-tailed tests were used.

### Mite-avoidance behavior

Another possible confounding feature to testing pupal selection, even given equal-exposure to mites, is the ability of the pupa to prevent the attachment of mites by body movements. For example, if female pupae less effectively dislodge mites, they may accumulate more mites even if mites are equally attracted to male and female pupae. Also, if individuals reared on the reduced food level less effectively dislodge mites, they too may accumulate more mites even if mites are equally attracted to pupae of different sizes.

The ability of pupae to prevent mite attachment was tested by observing the detailed interactions between pupae of *A. crucians* and larvae of *A. novimarsallae* in the following manner. While still in the constant-



TABLE I. *Average number of mites on the 4 normal male and 4 normal female pupae in each of 8 trials. Ranges are given in parentheses.*

Trial	Males		Females	
	Avg. no. mites	Avg. pupal dry weight (mg)	Avg. no. mites	Avg. pupal dry weight (mg)
1	2.25 (0-5)	0.456 (0.392-0.504)	4.75 (0-8)	0.597 (0.544-0.644)
2	4.25 (1-12)	0.485 (0.446-0.512)	11.50 (6-16)	0.583 (0.530-0.620)
3	3.75 (2-6)	0.425 (0.364-0.466)	4.50 (1-8)	0.569 (0.500-0.598)
4	0.25 (0-1)	0.456 (0.426-0.522)	2.25 (0-6)	0.623 (0.584-0.668)
5	3.25 (2-5)	0.499 (0.456-0.542)	7.00 (2-10)	0.536 (0.350-0.636)
6	1.50 (0-5)	0.440 (0.378-0.502)	3.00 (0-6)	0.521 (0.498-0.560)
7	5.50 (2-11)	0.435 (0.404-0.480)	8.75 (7-12)	0.588 (0.572-0.614)
8	16.25 (5-23)	0.408 (0.350-0.442)	24.00 (14-44)	0.594 (0.580-0.624)

TABLE II. *Average number of mites on the 4 normal male and 4 small female pupae in each of 8 trials. Ranges are given in parentheses.*

Trial	Males		Females	
	Avg. no. mites	Avg. pupal dry weight (mg)	Avg. no. mites	Avg. pupal dry weight (mg)
1	2.00 (1-3)	0.448 (0.408-0.480)	2.75 (0-5)	0.280 (0.270-0.292)
2	13.75 (8-18)	0.443 (0.410-0.472)	24.25 (17-31)	0.271 (0.226-0.308)
3	9.00 (3-22)	0.452 (0.436-0.468)	9.25 (2-17)	0.300 (0.276-0.330)
4	4.00 (2-7)	0.413 (0.358-0.446)	47.00 (9-149)	0.260 (0.246-0.272)
5	1.00 (0-2)	0.422 (0.402-0.444)	2.25 (0-5)	0.322 (0.294-0.362)
6	6.50 (3-9)	0.409 (0.392-0.436)	10.50 (5-20)	0.312 (0.280-0.358)
7	1.50 (0-5)	0.381 (0.340-0.400)	8.25 (3-16)	0.279 (0.242-0.308)
8	16.50 (9-39)	0.391 (0.330-0.426)	14.00 (7-26)	0.284 (0.258-0.308)

environment rearing chamber, a pupa was transferred from its holding container to 1 ml of aquarium water in 1 of the 2-cm-diameter depressions of a white porcelain depression plate (112 × 92 × 7 mm) where it remained for at least an hour. Then a mite was added, the depression plate was immediately moved from the rearing chamber to a dissecting-microscope stage, and the pupa and mite were observed for the next 10 min. The heavy porcelain plate minimized the temperature change experienced by the pupa during the 10-min observation period after the move from rearing chamber (28 C) to microscope stage (approximately 26 C). This procedure was repeated on other pupa-mite pairs until 20 normal and 20 small pupae of each sex had been tested. Each pupa and mite were used only once. The entire depression containing the pupa-mite pair was visible through the microscope with only background lighting (overhead fluorescent lights and sunlight), so observations could be made without moving the porcelain plate and without possible interference from microscope lamp illumination.

All tests were run during the daylight hours within a 3-wk period. Pupae used were reared as mentioned previously and were from 24 to 36 hr old (as pupae). Data recorded in each 10-min period were the number of mite-pupa contacts resulting in an immediate avoidance reaction by the pupa (causing a break in mite-pupa contact), the number of dislodgements of an attached mite, the total duration of mite attachments, and the number of pupae with attached mites at the end of the 10-min observation period.

The difference between groups in the number of pupae with attached mites at the end of the 10-min observation period was tested with the chi-square test for 2 independent samples. The difference between groups in the number of attachment-avoidance responses per mite contact, which equals the number of dislodgements and immediate avoidance reactions divided by the number of mite contacts, was tested with the Mann-Whitney *U*-test. The difference between groups in the

average duration of mite attachments was tested also with the Mann-Whitney *U*-test. (Each mite contacting a pupa contributed an attachment time; mites immediately shaken loose contributed 0 min. Thus, the average duration of mite attachments equals the total duration of mite attachments divided by the number of mite contacts.)

The null hypothesis in all mite-avoidance tests was that 2 groups of pupae equally prevent mite attachment. The alternate hypothesis in all cases specified a direction for the difference, so 1-tailed tests were used.

## RESULTS

### Pupal duration

The number of hours males and females spent in the pupal stage was not significantly different ( $P > 0.15$ ). The means plus or minus the standard errors were  $38.00 \pm 0.12$  hr in the 29 males and  $38.18 \pm 0.15$  hr in the 39 females.

### Equal-exposure tests

When male and female pupae were matched and exposed equally to mites, female pupae bore significantly more mites (Tables I-III). Because the 5 aquaria contained different numbers of mite larvae at any one time during the experiments, valid comparisons of pupal mite numbers can be made only within a trial. In experiment 1 comparing normal pupae, females had more mites in all 8 trials ( $P = 0.004$ , Table I); in experiment 2 comparing normal male and small female pupae, females had more mites in 7 of 8 trials ( $P = 0.027$ , Table II); and in experiment 3 comparing small male and normal female pupae, females

TABLE III. Average number of mites on the 4 small male and 4 normal female pupae in each of 8 trials. Ranges are given in parentheses.

Trial	Males		Females	
	Avg. no. mites	Avg. pupal dry weight (mg)	Avg. no. mites	Avg. pupal dry weight (mg)
1	0.25 (0-1)	0.302 (0.286-0.308)	0.50 (0-1)	0.549 (0.514-0.592)
2	0.25 (0-1)	0.277 (0.242-0.304)	3.25 (0-9)	0.543 (0.490-0.608)
3	4.75 (2-11)	0.250 (0.238-0.270)	9.75 (8-12)	0.522 (0.454-0.568)
4	4.25 (1-7)	0.237 (0.224-0.252)	3.25 (0-6)	0.450 (0.400-0.506)
5	2.75 (1-4)	0.242 (0.232-0.268)	2.50 (1-4)	0.531 (0.506-0.554)
6	6.25 (3-12)	0.253 (0.230-0.290)	12.25 (7-19)	0.499 (0.412-0.556)
7	4.25 (2-10)	0.235 (0.182-0.276)	6.00 (1-12)	0.560 (0.526-0.594)
8	2.75 (0-6)	0.241 (0.220-0.252)	6.25 (4-9)	0.547 (0.502-0.584)

TABLE IV. Average number of mites on the 4 normal and 4 small male pupae in each of 8 trials. Ranges are given in parentheses.

Trial	Normal males		Small males	
	Avg. no. mites	Avg. pupal dry weight (mg)	Avg. no. mites	Avg. pupal dry weight (mg)
1	0.75 (0-2)	0.401 (0.366-0.424)	1.75 (0-4)	0.254 (0.214-0.284)
2	2.00 (0-6)	0.396 (0.340-0.438)	3.50 (0-10)	0.234 (0.142-0.322)
3	5.75 (2-9)	0.397 (0.372-0.450)	3.25 (1-6)	0.263 (0.232-0.292)
4	1.00 (0-3)	0.408 (0.396-0.438)	14.00 (4-34)	0.265 (0.196-0.346)
5	4.00 (0-14)	0.411 (0.314-0.498)	1.50 (0-5)	0.242 (0.232-0.256)
6	6.00 (4-8)	0.434 (0.402-0.452)	7.00 (2-11)	0.274 (0.244-0.302)
7	2.00 (0-5)	0.412 (0.366-0.474)	1.50 (0-3)	0.249 (0.212-0.270)
8	13.00 (2-17)	0.419 (0.406-0.440)	11.25 (5-25)	0.222 (0.208-0.236)

had more mites in 6 of 8 trials ( $P = 0.039$ , Table III). Thus, mites attached in greater numbers to female pupae irrespective of the size of male and female pupae in an experiment. In experiments 4 and 5, mite numbers were not significantly higher on larger than smaller pupae of the same sex (in males,  $P = 0.473$ , Table IV; in females,  $P = 0.230$ , Table V).

#### Mite-avoidance behavior

Nine of the 20 normal male pupae tested bore a mite at the end of the 10-min observation period, and 8 of the 20 normal female pupae tested bore a mite. This difference was not significant ( $P > 0.35$ ). Also, 10 of the 20 small male pupae tested bore a mite at the end of the 10-min observation period, and 7 of the 20 small female pupae tested bore a mite. The difference between normal and small pupae of the same sex was not significant ( $P > 0.25$  in males and  $P > 0.35$  in females). The number of pupae bearing a mite in each experimental group is equal to the difference between the number of mite contacts and the number of dislodgements and immediate avoidance reactions (Table VI).

Normal male and female pupae did not differ significantly in the number of attachment-avoidance responses per mite contact ( $P > 0.10$ ). Normal males prevented attachment after 138/147 or 94% of the mite contacts, and normal females prevented attachment after 169/177 or 95% of the mite contacts (Table VI). Likewise, small and

normal pupae of the same sex did not differ significantly in the number of attachment-avoidance responses per mite contact ( $P > 0.10$  in both males and females). Small males prevented attachment after 140/150 or 93% of the mite contacts, and small females prevented attachment after 173/180 or 96% of the mite contacts (Table VI). The individual pupal values in all 4 groups ranged from 0 to 100%.

Normal male and female pupae did not differ significantly in the average duration of mite at-

TABLE V. Average number of mites on the 4 normal and 4 small female pupae in each of 8 trials. Ranges are given in parentheses.

Trial	Normal females		Small females	
	Avg. no. mites	Avg. pupal dry weight (mg)	Avg. no. mites	Avg. pupal dry weight (mg)
1	2.25 (0-4)	0.588 (0.566-0.640)	1.25 (0-3)	0.295 (0.240-0.350)
2	2.75 (0-7)	0.553 (0.462-0.618)	6.25 (0-12)	0.301 (0.272-0.346)
3	56.00 (27-106)	0.663 (0.590-0.786)	23.00 (14-37)	0.293 (0.234-0.348)
4	4.75 (1-10)	0.498 (0.464-0.538)	2.50 (1-5)	0.239 (0.202-0.264)
5	5.00 (3-9)	0.540 (0.492-0.576)	4.25 (1-8)	0.303 (0.268-0.342)
6	1.00 (0-2)	0.526 (0.470-0.576)	3.00 (0-6)	0.280 (0.242-0.334)
7	16.00 (5-29)	0.563 (0.504-0.660)	4.00 (1-8)	0.286 (0.248-0.324)
8	5.50 (4-6)	0.540 (0.484-0.600)	6.50 (2-8)	0.274 (0.200-0.376)



TABLE VI. Summary of mite-avoidance observations on the 20 pupae in each of 4 experimental groups of pupae.

	Total no. dislodgements and immediate avoidance reactions	Total duration (min) of mite attachments	Total no. mite contacts
Normal males	138	96.27	147
Normal females	169	90.95	177
Small males	140	81.87	150
Small females	173	62.26	180

tachment ( $P > 0.10$ ). Mites remained attached to normal males for an average of 96.27/147 or 0.65 min (individual pupal values ranged from 0 to 9.35 min) and to normal females for an average of 90.95/177 or 0.51 min (individual pupal values ranged from 0 to 9.52 min) (Table VI). Similarly, small and normal pupae of the same sex did not differ significantly in the average duration of mite attachment ( $P > 0.10$  in males and  $P > 0.05$  in females). Mites remained attached to small males for an average of 81.87/150 or 0.55 min and to small females for an average of 62.26/180 or 0.35 min (individual values ranged from 0 to 10 min in both sexes of small pupae) (Table VI).

Thus, normal males are not more adept at preventing mite attachment than are normal females; in fact, the low but still nonsignificant  $P$  values in 2 of the above tests of normal males vs. females were due to the generally better performance of female rather than male pupae in preventing mite attachment. Also, normal pupae are not more adept at preventing mite attachment than are small pupae of the same sex; the low but still nonsignificant  $P$  values in several of the above tests of normal vs. small pupae were due to the generally better performance of small rather than normal pupae of the same sex in preventing mite attachment. The lower food level that produced small pupae did not reduce the pupa's ability to prevent mite attachment.

Further support for the similar mite-avoidance behavior of *A. crucians* pupae, irrespective of their sex or size as affected by food level, is the nonsignificance ( $0.40 > P > 0.05$ ) in tests between all other possible pairings of the 4 groups of pupae.

#### DISCUSSION

Higher parasitic mite abundances on female hosts result from nonpreferential or preferential modes of transmission. Nonpreferential modes

include temporal overlap, as found by Smith and McIver (1984a, 1984c), and also exposure time, i.e., females may spend more time than males in the pupal stage and so would be susceptible to parasitism longer. In addition, a nonpreferential mode may result from the differential ability of pupal sexes to avoid or dislodge mites; pupae may be equally attractive to mites, but if females are less effective at preventing mite attachment, they will bear higher mite abundances. A preferential mode is the actual selecting of females over males, e.g., given equal mite exposure to pupae of each sex and equal abilities of each sex in preventing mite attachment, mites would attain higher abundances on female than male pupae.

Because of the nearly continuous emergence of each sex of *A. crucians* in the region of study, temporal overlap cannot explain the higher abundances of *A. novimmarshallae* on female hosts. Also, the pupal durations of males and females do not differ significantly, and female pupae are not less effective at preventing mite attachment. Thus, the modes of nonpreferential transmission mentioned are inoperative.

A preferential mode of transmission appears to explain the higher mite abundances on female *A. crucians*. In equal-exposure tests, female pupae consistently carried higher mite numbers. This trend was not changed when female pupal body size was smaller than male pupal body size, and in other experiments, pupal body size alone did not significantly affect attached mite numbers or mite-avoidance behavior. Mite larvae of *A. novimmarshallae* somehow detect and attach preferentially to female pupae of their host species *A. crucians*. The fitness advantage accruing to mites on females is likely due to the higher probability of returning to water as the mosquito oviposits.

The preference for female pupae is relative, however; many mites still attach to male pupae. Attachment to males despite the mite's preference for and apparent ability to detect females suggests that parasitizing the less attractive sex has not been strongly selected against over evolutionary time. One possible explanation lies in the limited life span of an unattached mite larva, the probability of pupal encounter, and the probability of returning to water from a male host. If the rate of pupal encounter is low enough and the probability of returning to water from a male host is greater than 0, a mite may be more successful remaining on a male rather than con-

tinuing to search because the mite may starve before discovering a female.

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## PATHOGENICITY OF *TRITRICHOMONAS MOBILENSIS*: SUBCUTANEOUS INOCULATION IN MICE

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**ABSTRACT:** The standard "subcutaneous mouse assay" was used to investigate the inherent pathogenicity of *Tritrichomonas mobilensis*, an intestinal parasite of squirrel monkeys. C57Bl/6 mice given subcutaneous bilateral inocula of *T. mobilensis* died by day 4 postinoculation with lesions too small to be measured. Control mice similarly inoculated with pathogenic and nonpathogenic strains of *Trichomonas vaginalis* survived the challenge and produced lesions on day 6 with mean volumes in agreement with previous reports. CD1 mice similarly inoculated with standard and double doses of trichomonads (*T. mobilensis*) again produced small lesions. CD1 mice inoculated at double dosage were moribund or dead on days 5 and 6, respectively, postinoculation. Necropsies were performed on dead and sacrificed mice. Tissues were obtained from internal organs for histology and culture. Unexpectedly, trichomonads were cultured from liver and lung of C57Bl/6 mice at the standard level of inoculation and liver, lung, and spleen of CD1 mice at the higher level of inoculation.

Although trichomonads are normally considered surface-dwelling noninvasive organisms, the penetration of trichomonads to deep tissues is not without precedent. *Tritrichomonas foetus* and *Trichomonas gallinae* are known to invade tissues of their respective hosts. *Trichomonas vaginalis* has been demonstrated in subepithelial areas of both the prostate gland and cervix of humans. The ability of several species of trichomonads to invade tissues and/or migrate to other sites in their hosts suggests a need for revision of the concept of trichomonads as strictly lumen or surface-dwelling parasites.

A new species of trichomonad, *Tritrichomonas mobilensis*, isolated from rectal swabs of squirrel monkeys in the Primate Research Laboratory of the University of South Alabama, Mobile, Alabama, was recently described (Culberson et al., 1986). The ubiquitous occurrence of *T. mobilensis* in this and other squirrel monkey colonies (Pindak et al., 1985, 1987) suggested a need for virulence assessment of this parasite.

A number of systems for the estimation of virulence/pathogenicity of trichomonads have been proposed (Honigberg et al., 1964, 1966; Teras and Roigas, 1966; Warton and Honigberg, 1980, 1983). One of the most useful, albeit technically involved, is the "subcutaneous mouse assay." Despite some objections (Alderete, 1983), this assay repeatedly has been shown to correlate most closely with observed pathologic effects in the natural host. We decided, therefore, to investigate the pathogenicity of *T. mobilensis* using this assay and minor modifications thereof.

### MATERIALS AND METHODS

#### Cultivation of trichomonads

*Tritrichomonas mobilensis* (ATCC 50116) was grown in modified Diamond's medium (Diamond, 1957), pH

7.0, with 10% heat-inactivated horse serum and without agar at 37 C. Two strains of *Trichomonas vaginalis* of known pathogenicity, Balt 42 and JH31A (courtesy of Dr. B. M. Honigberg, University of Massachusetts at Amherst, Amherst, Massachusetts), were grown in similar medium of pH 6.2 to be used as controls. Cells were harvested for inoculation into mice from 24- to 36-hr cultures by centrifugation at 1,000 g for 10 min, washed once in fresh Diamond's medium, counted on a Neubauer hemacytometer, and diluted to appropriate concentrations in thioglycollate medium with 10% heat-inactivated horse serum.

#### Strains of mice used

Two separate experiments were conducted. The first used the standard highly inbred C57Bl/6 CRK mice; the second used outbred (CD1) mice.

**Experiment 1:** Twenty-seven 9-wk-old female C57Bl/6 CRK mice were inoculated subcutaneously with  $9 \times 10^5$  *T. mobilensis* organisms in 0.5 ml thioglycollate/10% heat-inactivated horse serum in both flanks as described previously (Honigberg et al., 1984). Twenty-five control animals were similarly inoculated with 2 strains of *T. vaginalis* of known pathogenicity for mice: the highly pathogenic Balt 42 (15 animals) and the relatively benign JH31A (10 animals). To control for sterility, blood agar plates were inoculated with all flagellate suspensions before and after administration to mice. At least 1 mouse inoculated with *T. mobilensis* was killed each day postinoculation. Material for histology and culture was obtained from the sites of inoculation and from liver and lung. For culture, ~1-mm<sup>3</sup> pieces of tissue were immersed in Diamond's medium and incubated at 37 C for up to 10 days. For histology, tissues were fixed in buffered formalin, embedded in paraffin by standard procedure, sectioned, and stained with hematoxylin and eosin. Care was taken to avoid contamination by material from

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TABLE I. Experiment 1, lesion volume—day 6.

Species/strain	n*	Mean volume (mm <sup>3</sup> )	Range
<i>T. vaginalis</i> /Balt42	18	180.0	140.5–210.0
<i>T. vaginalis</i> /JH31A	15	81.5	55.0–100.5
<i>T. mobilensis</i> /ATCC50116	All mice dead by day 4, lesions too small to be measured		

\* Number of lesions measured. Some lesions drain by day 6.

the sites of inoculation during the collection of internal organ tissue.

Lesions produced with Balt 42 and JH31A were measured on day 6 postinoculation in accordance with standard procedure as outlined elsewhere (Honigberg et al., 1984).

**Experiment 2:** Six CD1 mice (referred to hereafter as LDM) were inoculated as above with  $1 \times 10^6$  *T. mobilensis*/flank. Another 6 CD1 mice (referred to hereafter as HDM) were inoculated similarly but with  $2 \times 10^6$  organisms/flank. A single control mouse was inoculated with 0.5 ml sterile thioglycollate/10% inactivated horse serum per flank. Assay for sterility of inocula was performed as above. No contaminants were present. Mice were killed, 1 at each level of inoculation per day for 6 days. The control animal was killed on day 6. Tissues from liver, lung, spleen/pancreas, and material from the lesion were collected and prepared as above for histology and culture. Fecal samples were collected for culture on days 5 and 6.

## RESULTS

### Experiment 1

Lesion volumes (Table I) in control animals (Balt 42, JH31A) agree with those previously reported (Kuczynska et al., 1984). All mice inoculated with *T. vaginalis* strains survived. In contrast, all 27 mice inoculated with *T. mobilensis* were dead by the end of the fourth day postinoculation with lesions too small to be measured accurately. After day 1, moribund animals were sacrificed for histology and culture. Thus, only on day 1 was an apparently healthy mouse sacrificed. Deaths following day 1 were: day 2: 1 dead, 1 sacrificed; day 3: 8 dead, 2 sacrificed; day 4: 10 dead, 4 sacrificed. Moribund mice displayed partial paralysis of hindquarters, shallow breathing, matted coats, and listlessness. Sites of inoculation, liver, and lung were cultured (Table II). All samples from inoculation sites were positive for trichomonads. In several mice *T. mobilensis* was isolated on day 3 from the lung and on day 4 from liver and lung.

Microscopic study of lesions 24 hr postinoculation revealed trichomonads within an ill-defined oval area extending into subcutaneous muscle and fat (Fig. 1). A mild inflammatory

TABLE II. Experiment 1, culture results.

Species/strain		Lesion	Liver	Lung
<i>T. vaginalis</i> /Balt42	(day 6)	+	—	—
<i>T. vaginalis</i> /JH31A	(day 6)	+	—	—
<i>T. mobilensis</i> /ATCC50116	(day 1)	+	ND*	ND
	(day 2)	+	ND	ND
	(day 3)	+	—	+
	(day 4)	+	+	+

\* ND, not done.

response consisting of occasional neutrophils and lymphocytes was present within this injection pocket, however, a reactive mantle of neutrophils surrounded the mass of trichomonads. Adjacent adipose tissue contained mild infiltration of neutrophils, occasional lymphocytes, and few trichomonads. Marked hyperemia was present within adjacent tissue and many blood vessels contained marginated and migrating leukocytes. An occasional trichomonad was observed within dilated lymphatics.

Lung tissue revealed mild infiltration of thickened alveolar walls with neutrophils and lymphocytes. There was also pulmonary congestion, with patchy areas of edema and fibrin deposition in some alveolar spaces.

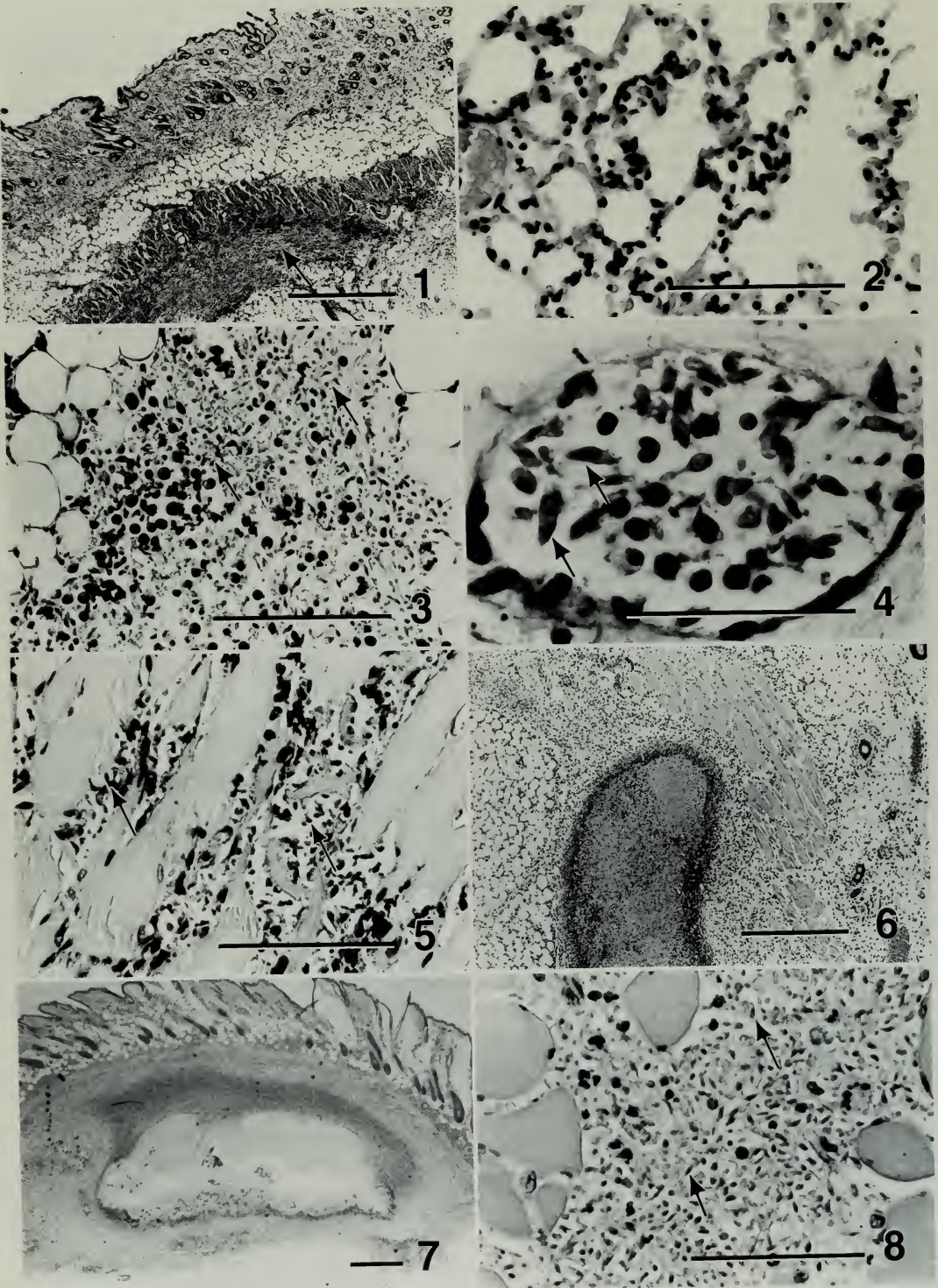
Splenic changes consisted of patchy hemosiderosis and diffuse congestion.

At 48 hr postinoculation, numerous organisms were present within the injection site as well as both the dermis and subcutaneous tissues. The neutrophilic mantle was moderately decreased compared to the cellular response at 24 hr. A mild neutrophilic response was observed in arterioles, venules, and veins in the adjacent subcutaneous tissue.

Moderate thickening of pulmonary alveolar walls was observed in lung tissue with congestion and mild infiltration of pulmonary interstitium by neutrophils, lymphocytes, and occasional macrophages. Multiple petechiae were also present. The pulmonary interstitial tissue had a higher cellular and nuclear density than the pulmonary tissue at 24 hr (Fig. 2). Mild hemosiderosis was again observed in splenic tissue.

At 72 hr postinoculation the pocket of trichomonads was surrounded by increased numbers of lymphocytes, macrophages, and decreased numbers of neutrophils compared to 48 hr postinoculation. There was separation of collagen and muscle fibers with fragmentation, hyalinization, and vacuolization of the sarcolemma. Numerous organisms were observed surrounding muscle fibers and within subcutaneous tissues.





FIGURES 1-8. Histologic sequelae of subcutaneous inoculation of *T. mobilensis* into mice. 1. Poorly defined trichomonad injection pocket (arrow) 24 hr postinoculation (PI), C57Bl/6 mouse, hematoxylin & eosin (H&E). Bar = 0.5 mm. 2. Increased pulmonary interstitial nuclear and cellular density 48 hours PI, C57Bl/6 mouse (H&E). Bar = 100  $\mu$ m. 3. Trichomonads packed within injection site infiltrating subcutaneous adipose tissue



Histopathologic changes in the lung at 72 hr consisted of septal wall thickening with moderate congestion. Petechiae and patchy areas of edema with fibrin deposition were observed.

Splenic changes included marked congestion with moderate splenic hemosiderosis.

At 96 hr postinoculation the injection site was packed with trichomonads and debris (Fig. 3), which infiltrated into lymphatics (Fig. 4) as well as adjacent subcutaneous and dermal tissue. Approximately one-fourth of the injection pocket contained cellular and nuclear debris with a small mantle of inflammatory cells surrounding trichomonads. Trichomonads were present beyond the initial pocket and extended into the subcutaneous and dermal tissue. Multifocal muscle fiber necrosis and degeneration (Fig. 5) were again observed in the subcutaneous tissues with a relative increase in macrophages.

Pulmonary lesions were comparable to those seen at 72 hr postinoculation.

Splenic changes were most prominent at day 4, consisting of marked congestion and hemosiderosis. Hyperplasia of lymphocytes surrounding periarteriolar sheaths was also observed.

## Experiment 2

Lesion development in outbred (CD1) mice inoculated with  $1 \times 10^6$  or  $2 \times 10^6$  *T. mobilensis*/flank was similar to that noted in experiment 1 (i.e., minimal). Each mouse inoculated with a total of  $2 \times 10^6$  organisms appeared to be healthy when killed. Each mouse inoculated with  $4 \times 10^6$  organisms was healthy on days 1–4. On day 5 the animal killed was exhibiting symptoms similar to those noted in experiment 1 and the remaining animal at the higher level of inoculation died following similar symptoms on day 6.

Culture results for experiment 2 are presented in Table III. No trichomonads were isolated from the internal organs of mice inoculated with  $2 \times 10^6$  trichomonads/animal, whereas with the higher level inoculum, trichomonads were isolated from lung and spleen on day 4, and from liver, lung, and pancreas on days 5 and 6. Likewise, lesion material was positive for tricho-

monads at both levels of inoculation on days 2–5 as well as at the higher level of inoculation on day 1. Material from the sites of inoculation at both levels was negative for trichomonads on day 6. Cultures of fecal material on days 5 and 6 were also negative.

Lesions in CD1 mice at 24 hr postinoculation revealed a typical neutrophilic response adjacent to the pocket of trichomonads. Microthrombi were present within the adjacent tissue arterioles. Trichomonad injection pockets were multilocular with numerous neutrophils intermixed with organisms in both high- and low-dose groups.

At 48 hr postinoculation the progression of the neutrophilic response was similar in both groups.

At 72 hr postinoculation a well-formed and organizing abscess was observed within the injection site of the LDM. The inflammatory response extended deep into both the dermis and subcutaneous tissue.

Organisms in the LDM were more difficult to find than those observed in the injection site at 24 hr. The injection site in the HDM was multilocular and contained well-defined trichomonads. The inflammatory response in this mouse was less extensive than in the LDM and exhibited less fibroplasia.

At 96 hr postinoculation the LDM had a well-developed abscess with a fibrous capsule (Fig. 6). Occasional trichomonads were observed within the adjacent tissues but few intact organisms were observed within the injection site. An increase in the number of macrophages and lymphocytes was observed compared to 72 hr. The HDM had a mantle of neutrophils surrounding a large pocket of intact organisms.

Within the adjacent tissue, multifocal areas of necrosis were observed and neutrophils persisted within the dermis and subcutaneous fat layer. Moderate fibroblast proliferation was observed but was considerably less than that observed in the LDM response.

At 5 days postinoculation a well-delineated abscess with a central liquefactive necrotic core was observed in the LDM (Fig. 7). Trichomonads were not discernible in the injection site or in adjacent tissues. The inflammatory response

← 96 hr PI (area indicated by arrows), C57Bl/6 mouse (H&E). Bar = 100  $\mu$ m. 4. Infiltration of trichomonads (arrows) into lymphatics 96 hr PI, C57Bl/6 mouse (H&E). Bar = 50  $\mu$ m. 5. Focal areas of cutaneous muscle fiber necrosis with trichomonads (arrows) 96 hr PI, C57Bl/6 mouse (H&E). Bar = 100  $\mu$ m. 6. Well-developed abscess 96 hr PI, low-dose CD1 mouse (H&E). Bar = 0.5 mm. 7. Abscess with central liquefactive core and rim of inflammatory cells 5 days PI, low-dose CD1 mouse (H&E). Bar = 0.5 mm. 8. Mass of trichomonads (arrows) extending into and investing cutaneous muscle 5 days PI, high-dose CD1 mouse (H&E). Bar = 100  $\mu$ m.



TABLE III. Experiment 2, culture results from CD1 mice inoculated with *T. mobilensis*.

Days post-inoculation	Organisms/mouse*	Liver	Lung	Spleen/pancreas	Lesion	Fecal
1	2 × 10 <sup>6</sup>	—	—	—	—	ND†
	4 × 10 <sup>6</sup>	—	—	—	+	ND
2	2 × 10 <sup>6</sup>	—	—	—	+	ND
	4 × 10 <sup>6</sup>	—	—	—	+	ND
3	2 × 10 <sup>6</sup>	—	—	—	+	ND
	4 × 10 <sup>6</sup>	—	—	—	+	ND
4	2 × 10 <sup>6</sup>	—	—	—	+	ND
	4 × 10 <sup>6</sup>	—	+	+	+	ND
5	2 × 10 <sup>6</sup>	—	—	—	+	—
	4 × 10 <sup>6</sup>	+	+	+	+	—
6	2 × 10 <sup>6</sup>	—	—	—	—	—
	4 × 10 <sup>6</sup>	+	+	+	—	—
	Control	—	—	—	—	—

\* Total inoculum injected bilaterally (0.5 ml fluid thioglycollate containing 10% heat-inactivated horse serum and ½ total organisms per flank).

† ND, not done.

was more monocytic, consisting of lymphocytes and macrophages with fewer numbers of neutrophils and occasional plasma cells. A larger number of fibroblasts was seen in the adjacent tissue. The HDM exhibited a mass of trichomonads extending into subcutaneous muscle tissue (Fig. 8), fat, and dermis with minimal neutrophilic inflammatory response. Trichomonads were disseminated in adjacent tissue. The injection site at the higher dose contained mostly degenerating cells and trichomonads.

On day 6 postinoculation the microscopic appearance of tissues in the LDM was similar to day 5 but with more collagen layers, fibroblasts, and chronic inflammatory cells. The HDM tissues also had an appearance similar to that observed on day 5 with the exception of more widespread trichomonad dissemination and more foci of degenerating organisms. The lesion at this site on day 6 was the least conspicuous of the group.

The control mouse on day 6 had micro- and macrofoci of collagen fiber separation and edema within the injection site. Tissue adjacent to the inoculation site exhibited a minimal inflammatory response consisting of a few macrophages within interstitial and subcutaneous tissue. The lesion site in the control contained only occasional macrophages, lymphocytes, and neutrophils.

## DISCUSSION

The behavior of *T. mobilensis* in the subcutaneous mouse assay is in striking contrast to that exhibited by all other tested trichomonads. In our experiment, 2 established and well-characterized strains of *Trichomonas vaginalis* pro-

duced lesions comparable to those observed by other authors (Kuczyńska et al., 1984). In contrast, *T. mobilensis* proceeded from the inoculation site into adjacent tissues and migrated, apparently via lymphatics, to other organs of the body. These observations have relevance to the biology of trichomonads in general.

The failure of inoculated mice, especially in experiment 1, to produce measurable lesions may have been a result of their apparent inability to mount an aggressive local inflammatory response to the trichomonad inoculum. However, lesions from mice in experiment 2 that gave the appearance, at least histologically, of the more usual inflammatory response were likewise not easily measurable. It may be that the migration of trichomonads into the surrounding tissues resulted in the dispersal of stimuli that normally contribute to lesion volume in this assay. The question of the immunocompetence of C57Bl/6 mice to respond to subcutaneous inoculation of this particular trichomonad species also arises. All C57Bl/6 mice died by day 4 with migration of trichomonads to internal organs at the lower level of inoculation. Migration to internal organs in CD1 mice was observed only at higher (doubled) levels of inoculation and death was observed only on day 6. These observations support the conclusion that C57Bl/6 mice, for unknown reasons, are unable to mount as effective an immune response to inoculation to *T. mobilensis* as observed in CD1 mice.

The cultivation of trichomonads from the liver of animals in experiment 1 (day 4) could not be correlated with histopathologic changes at that site. However, the cultivation of trichomonads

from lung on days 3 and 4 was accompanied by progressive thickening of alveolar walls and an increase in pulmonary interstitial cellular density. The migration of trichomonads into muscle fibers surrounding the site of inoculation with accompanying muscle fiber degeneration may account in part for the observed paralysis of hindquarters in C57Bl/6 mice (experiment 1). Up to day 6 the histopathology of lesions in experiment 2 is comparable to that observed by Frost and Honigberg (1962) for *T. vaginalis* and *T. gallinae*.

Several etiologies may be considered in the death of mice on day 4 in experiment 1 and on day 6 in HDM in experiment 2. A systemic parasitemia accompanying migration of trichomonads to internal organs would be expected to have potential for producing debilitating effects in experimental hosts. Additionally, the splenic hemosiderosis observed, particularly in experiment 1, indicates destruction of erythrocytes. Thickening of alveolar walls suggests the possibility of pulmonary compromise. Additionally, this argues that the highly inbred C57Bl/6 mice may have unreported immunologic peculiarities that contributed to the dramatic death rates observed in experiment 1.

The value of any assay for pathogenicity of a potential pathogen lies in the correlation of experimental results with the clinical picture in the natural host. The interaction of *T. mobilensis* with squirrel monkey intestinal epithelium is currently under investigation. There is, however, at least one report of invasive trichomoniasis due to *Tritrichomonas* sp. in a Titi monkey (*Callicebus moloch*) (Bunton et al., 1983). In this report multiple irregular mucosal erosions were observed at the ileocecolic junction. Histologic analysis demonstrated organisms (*Tritrichomonas* sp.) present in ulcerated areas, submucosal foci, and lymphatics as well as within sinusoids and mesenteric lymph nodes. No pathogenic bacteria were cultured from fecal samples. The possibility of trichomonad migration to other body sites in natural hosts has not been evaluated.

Several species of trichomonads have now been reported to have at least limited invasive potential. Although *Trichomonas gallinae* normally inhabits the mouth, pharynx, esophagus, and crop of birds, virulent strains have been shown to invade tissues and migrate to other parts of the body including head and neck sinuses, the eye and brain, lungs, heart, liver, and pancreas (Ho-

nigberg, 1978). *Tritrichomonas foetus* may invade the placenta as well as developing bovine embryos and fetuses, resulting in abortion (Honigberg, 1978). The presence of *T. vaginalis* has been demonstrated in submucosal and stromal areas of the prostate gland (Gardner et al., 1986) as well as in subepithelial tissue in a case of trichomonal cervicitis (Gardner et al., 1987). Our present studies indicate remarkable invasive and disseminative capacity (probably via lymphatics) for *T. mobilensis* in mice. Corroborative evidence of clinical disease due to an invasive simian *Tritrichomonas* sp. has also been reported (Bunton et al., 1983). It may be suggested therefore that trichomonads, particularly ones known to be pathogenic, should not be categorically viewed as strictly lumen-dwelling or surface-dwelling organisms.

The present study is one component of a broadly based approach to elucidation of the pathogenicity of *T. mobilensis*. Further studies involve its effect on cell culture monolayers and on the natural host. The question of invasiveness of this and other trichomonad species is an issue of considerable interest and importance. Further studies will be required to determine the extent of this invasiveness in *T. mobilensis* and what, if any, effect associated migration may have on the health of the natural hosts. Natural invasiveness as a normal characteristic of trichomonads in general clearly deserves further consideration.

#### ACKNOWLEDGMENT

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## GROWTH OF PLEOMORPHIC *TRYPANOSOMA BRUCEI RHODESIENSE* IN IRRADIATED INBRED MICE

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**ABSTRACT:** It was shown that irradiation (650 rad) of 7 inbred strains of mice did not block the ability of *Trypanosoma brucei rhodesiense* to transform from the long slender (LS) to the short stumpy (SS) form or alter the plateau in parasitemia. In addition, it was observed that significant differences in parasitemia levels, in the rate of transformation from the LS to the SS form, as well as in the survival times occurred between the irradiated C3HeB/FeJ and several of the other strains. These differences in the nonspecific ability to control parasitemia appeared to be characteristic for each inbred strain of mice. The resistant strains generally had lower parasitemia than the susceptible strains. However, it was also shown that there is not a one-to-one correlation between the innate ability of a mouse strain to control its initial parasitemia, and the strain's ability to clear the parasitemia or increase its survival time. It was therefore concluded that the hypothesis which states that the ability of an animal to increase nonspecifically the rate of transformation, and therefore to lower the parasitemia, allowing intact animals to respond immunologically and survive longer is either incorrect or incomplete. The results further show that the ability of mice to clear their initial parasitemia by an antibody response is not necessarily correlated with their survival time. Therefore, this study suggests that factors other than an antibody response and the nonspecific control of parasitemia are important in resistance.

It has been shown by several investigators that different inbred strains of mice have varying degrees of resistance to a number of different strains of African trypanosomes (Levine and Mansfield, 1981; Murray et al., 1982; Greenblatt et al., 1984). In several publications, it has appeared that the C3HeB/FeJ strain is one of the more susceptible strains to the African trypanosomes, whereas the B10.A/SgSnJ and B10.Br/SgSnJ strains are among the more resistant. It has been assumed by most researchers that the difference observed in survival times is due to the ability of the various inbred strains to mount an effective immunoglobulin response, particularly an IgM response (Levine and Mansfield, 1984). There are, however, alternatives to this suggestion. For example, it is possible that the difference in survival times is not related to the immune response, but rather to the ability of the different inbred strains to adjust physiologically to the stress of infection. It has been shown that there are a number of physiological changes that occur in infected rodent models (Hall and Seed, 1984). It is, therefore, possible that survival times are based upon the different abilities of the inbred strains to maintain physiological homeostasis. This paper describes the growth of *Trypanosoma brucei rhodesiense* in 7 different strains of intact and irradiated inbred mice. It compares their individual parasitemias, the rates of morphological transition of trypanosomes from the long slender (LS)

to the short stumpy (SS) stage, and mouse survival times. It was suggested that nonresponder strains might be able to produce an immune-induced remission in parasitemia if the initial parasitemia was better controlled. It was also considered possible that some strains could truly be immunodeficient and be completely unable to respond to one or more trypanosome surface epitopes. Thus, the strains of intact animals that failed to clear the initial parasitemia were further examined for their ability to produce a decrease in parasitemia.

This report is the first systematic examination of the control of parasitemia in different inbred mouse strains infected with the African trypanosomes. More importantly, it is the first full study of the hypothesis that the innate factors that control the initial parasitemia are directly correlated with resistance to African trypanosomiasis. The ability of susceptible strains of inbred mice to respond with antibody to an actual trypanosome infection when their parasitemia is experimentally lowered was also investigated. Prior studies have only demonstrated that susceptible strains, such as the C3H strain, could respond to trypanosome extracts or irradiated trypanosomes, a very different phenomenon than being able to respond and clear their parasitemia during an actual infection.

### MATERIALS AND METHODS

#### Animals

The 7 inbred mouse strains (C3HeB/FeJ, B10.A/SgSnJ, B10.BR/SgSnJ, C3H/HeJ, AKR/J, DBA/2J,

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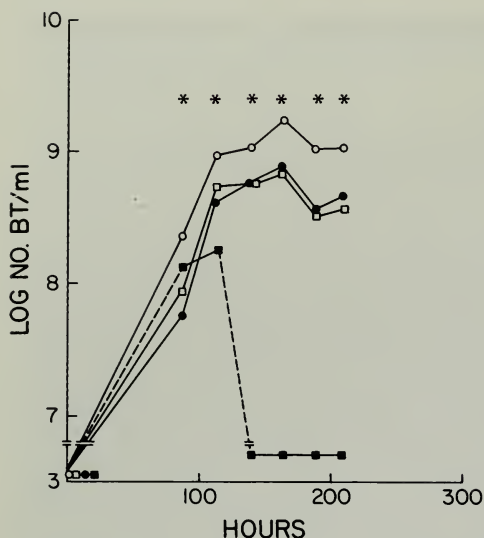


FIGURE 1. The growth of *T. b. r.* (LouTat-1) in 3 different female irradiated inbred mouse strains C3HeB/FeJ (○), B10.A/SgSnJ (●), and B10.BR/SgSnJ (□). The \* indicates time points at which the parasitemias in irradiated C3H mice were significantly higher (5% level) than either the irradiated B10.A or B10.BR mice. For comparison, the growth of *T. b. r.* (LouTat-1) in unirradiated B10.BR (■) is also shown for the first 9 days of infection.

C57Bl/6By) used in these studies were all obtained from Jackson Laboratories, Bar Harbor, Maine. They were selected because data on the resistance of these intact strains have previously been reported (Levine and Mansfield, 1981; Murray et al., 1982; Jones and Hancock, 1983; Greenblatt et al., 1984). The  $F_1$  offspring of a cross between the susceptible C3HeB/FeJ and the resistant B10.BR/SgSnJ strains were raised at UNC. Both male and female mice were independently compared. In addition, the randomly bred albino CD-1 mouse from a breeding colony at UNC was used. For comparative purposes all animals were age and sex matched. They were maintained under identical environmental conditions of temperature and lighting, and fed *ad libitum*. The irradiated animals were given a total body (midline) dose of 650 rad (650R) at a rate of approximately 120 rad/min from a  $^{137}\text{Cs}$  source, and infected 24 hr later. This protocol is basically identical to that described by other investigators (Balber, 1972; Luckins, 1972; Black et al., 1985). The inability of irradiated (650R) animals to respond immunologically to the trypanosomes was clearly demonstrated by their inability to significantly reduce their parasitemia at any time during the infection (Fig. 1). This is in sharp contrast to the unirradiated resistant animals that cleared their parasitemia. The only mechanism other than chemotherapy known to reduce and clear the parasitemia is through the action of antibody synthesized in response to the trypanosomes. Irradiated animals in the absence of the infection survive considerably longer than the infected irradiated animals (greater than 40 days).

## Trypanosomes

The monomorphic TXTat-1 clone of *T. b. gambiense* (*T. b. g.*, originally the Wellcome TS strain) has been previously described (Seed et al., 1984). The LouTat-1 clone of *T. b. rhodesiense* (*T. b. r.*) was obtained from Dr. J. Mansfield, and was originally derived from the EATRO 1886 strain. This clone is pleomorphic in mice.

## General

Mice were infected by the intraperitoneal route with  $5 \times 10^3$  washed, DEAE-cellulose-harvested cells suspended in HEPES (0.25 M, pH 7.4)-buffered complete RPMI-1640 culture medium with L-glutamine (Microbiological Associates, Maryland) plus 0.023 M  $\text{NaHCO}_3$ . The infections were monitored daily by examination of tail blood for parasitemia level. The blood was diluted in 1% glucose phosphate-buffered saline (0.025 M GPBS, pH 7.4) plus 1% sodium citrate, and the blood trypanosomes were counted with a hemacytometer. In addition, individual blood smears were prepared for each animal and stained with Giesma. The stained slides were examined for the various morphological types using the criteria of Wijers (1959). Except where the parasitemia level was low, in which case the entire slide was surveyed, at least 100 trypanosomes were examined for morphological type. In addition, the reproductive status of each cell was determined and scored. Cells were counted as reproducing if they contained 2 kinetoplasts and 1 nucleus, or 2 kinetoplasts and 2 nuclei. Thus, all animals were monitored daily for parasitemia level, for survival time, and for the morphological and reproductive status of the trypanosomes. The survival time and other parameters of the C3HeB/FeJ unirradiated strain were compared with the data from the other intact inbred strains by the Student's 2-tailed *t*-test. Similarly, the survival times and other parameters of the irradiated C3HeB/FeJ<sub>650R</sub> strain were also statistically compared with the data from the other irradiated strains. The results are reported as significant (S) at a probability of 5% or less.

## Immune response

Inbred mice were monitored for their ability to respond immunologically in 2 ways. The first was to determine if animals were able to produce a decrease in their parasitemia. As previously noted, in the absence of chemotherapy, significant decreases in parasitemia are always associated with an antibody response to the trypanosomes. Secondly, inbred animal strains such as the C3HeB/FeJ strain that failed to show any evidence of a decrease in parasitemia were then infected with the monomorphic TXTat-1 clone of *T. b. g.* A single cell of this clone will kill a mouse within 7 days, with no evidence of a plateau or decrease in parasitemia, and no evidence of any immune response. The mice were then treated with 2% difluoromethylornithine (DFMO) in their drinking water at 2 different parasitemia levels. The first treatment began when the parasitemia was just detectable at 43–45 hr, and the second at approximately log 7.2 trypanosomes/ml and at 60–65 hr. All animals were monitored for parasitemia for a total of approximately 10 days. A dramatic decrease in parasitemia during the DFMO treatment is believed to denote a specific im-

TABLE I. A comparison of differences in the resistance of irradiated or intact inbred male mouse strains.\*

Inbred strain	H-2 haplotype	Survival time $\pm$ SD (days)	P (5%)†	Parasitemia P (5%)‡	PI index (%)§	% Animals clearing parasitemia	Immune response capacity
C3HeB/FeJ	H-2 <sup>k</sup>	19.25 $\pm$ 4.44	—	—	—	0	+ (DFMO)
C3HeB/FeJ <sub>650R</sub>		9.40 $\pm$ 0.82	—	—	0	0	—
C3H/HeJ	H-2 <sup>k</sup>	23.80 $\pm$ 1.43	NS	—	—	0	NT
C3H/HeJ <sub>650R</sub>		10.50 $\pm$ 0.75	NS	NS	0	0	—
AKR/J	H-2 <sup>k</sup>	24.34 $\pm$ 3.62	S	—	—	18.2	+ (DFMO)
AKR/J <sub>650R</sub>		10.70 $\pm$ 2.53	NS	S	82.2	0	—
DBA/2J	H-2 <sup>d</sup>	28.04 $\pm$ 2.23	S	—	—	100	+
DBA/2J <sub>650R</sub>		12.30 $\pm$ 2.01	NS	NS	0	0	—
CD-1	ND	31.10 $\pm$ 8.20	S	—	—	16.7	NT
CD-1 <sub>650R</sub>		11.05 $\pm$ 3.60	NS	NS	17.0	0	—
C57Bl/6By	H-2 <sup>b</sup>	57.63 $\pm$ 12.90	S	—	—	100	+
C57Bl/6By <sub>650R</sub>		13.28 $\pm$ 2.32	S	S	59.2	0	—
B10.A/SgSnJ	H-2 <sup>a</sup>	32.68 $\pm$ 6.70	S	—	—	100	+
B10.A/SgSnJ <sub>650R</sub>		13.85 $\pm$ 2.27	S	S	98.7	0	—
B10.BR/SgSnJ	H-2 <sup>k</sup>	37.80 $\pm$ 10.44	S	—	—	100	+
B10.BR/SgSnJ <sub>650R</sub>		13.20 $\pm$ 2.62	S	S	66.1	0	—

\* All animals were infected with  $5 \times 10^3$  *T. b. r.* (LouTat-1).

† The survival time of the C3HeB/FeJ unirradiated strain was compared with the survival time of the other intact inbred strains by the Student's 2-tailed *t*-test. Similarly, the survival time of the C3HeB/FeJ irradiated strain was statistically compared with the survival time of the other irradiated inbred strains. NS denoted the values were not significant.

‡ Results are reported as significant (S) if the parasitemia for all the time points during the plateau period were different. See also Figure 1. NS denotes that none of the time points examined were statistically different from the C3HeB/FeJ<sub>650R</sub> animals at the 5% level.

§ PI index (%). See Materials and Methods.

|| + denotes a positive immune response by a decrease in parasitemia. DFMO in parentheses indicates mouse strains that required DFMO to control their parasitemia in order to observe this response; — would denote a true inability to respond to either LouTat-1 or TXTat-1. NT denotes not tested.

mune response. The 2 different parasitemias at which treatment was begun were used to measure the ability of an inbred strain to successfully respond to different parasitemias (or parasite dose levels).

In independent experiments, it was shown that TXTat-1-infected C3HeB/FeJ mice treated with DFMO responded with significant antibody titer to TXTat-1. At predetermined times mice were killed and bled by cardiac puncture for the collection of antisera. Antibody titers to the TXTat VAT's were measured by the agglutination reaction as previously described (Seed, 1978).

#### Parasitemia level

The parasitemia levels in an infected irradiated inbred strain during the plateau in parasitemia was compared (Fig. 1) with the parasitemia in infected irradiated C3HeB/FeJ mice at the same time points. These results were reported as significant (S) if the parasitemias for all the time points examined were different at the 5% level or less. Nonsignificant results denoted that none of the time points examined were statistically different from the C3HeB/FeJ animals at the 5% level. The results clearly showed that all the time points during the plateau phase were either statistically different from the C3HeB/FeJ control or they were not (Table I, column 5).

#### Parasitemia index (PI)

For comparative purposes a parasitemia index was also calculated. The daily differences between mice in the same experimental group were averaged for the plateau period. The difference between females of the

C3HeB/FeJ strain and females of the B10.BR/SgSnJ strain was taken as 100%, and was used for the denominator of the equation. The PI value is simply given in percent, and it is not intended to imply any statistical value.

$$PI (\%) = \frac{\frac{\text{Log}_{10} \text{ parasitemia inbred strain examined} - \text{Log}_{10} \text{ parasitemia C3HeB/FeJ control group}}{\text{Log}_{10} \text{ parasitemia B10.BR, female mice}} - \frac{\text{Log}_{10} \text{ parasitemia C3HeB/FeJ, female mice}}{\text{Log}_{10} \text{ parasitemia B10.BR, female mice}}}{\text{Log}_{10} \text{ parasitemia B10.BR, female mice} - \text{Log}_{10} \text{ parasitemia C3HeB/FeJ, female mice}} \times 100.$$

## RESULTS

Table I shows that the survival times of male mice from 7 inbred mouse strains were generally different. These results confirm and extend the prior observations of others (Levine and Mansfield, 1981; Murray et al., 1982; Jones and Hancock, 1983; Greenblatt et al., 1984). The only difference observed was the survival time of the AKR/J strain which in this study was  $24.3 \pm 3.6$  days, whereas Greenblatt et al. (1984) reported a survival time of over 30 days. They also showed that the AKR/J strain survived longer than the DBA/2J strain. In contrast, no significant differ-



TABLE II. *A comparison of differences in the resistance of male and female mice in 3 inbred mouse strains.\**

Inbred strain	Sex	H-2 haplotype	Survival time $\pm$ SD (days)	<i>P</i> (5%)†	Parasitemia <i>P</i> (5%)‡	Parasitemia index 650R (%)	% Animals clearing parasitemia
C3HeB/FeJ	F	H-2 <sup>k</sup>	20.40 $\pm$ 2.16 <sup>e</sup>	—	—	0	0
C3HeB/FeJ <sub>650R</sub>			10.67 $\pm$ 0.75	—	—	0	0
C3HeB/FeJ	M	H-2 <sup>k</sup>	22.88 $\pm$ 4.54	—	—	0	0
C3HeB/FeJ <sub>650R</sub>			11.00 $\pm$ 0	—	—	0	0
B10.A/SgSnJ	F	H-2 <sup>a</sup>	44.08 $\pm$ 7.00	S	S	154.0	100
B10.A/SgSnJ <sub>650R</sub>			13.38 $\pm$ 1.57	S	S	71.5	0
B10.A/SgSnJ	M	H-2 <sup>a</sup>	32.68 $\pm$ 6.70	S	S	71.5	100
B10.A/SgSnJ <sub>650R</sub>			13.85 $\pm$ 2.27	S	S	71.5	0
B10.BR/SgSnJ	F	H-2 <sup>a</sup>	66.30 $\pm$ 26.60	S	S	100.0	100
B10.BR/SgSnJ <sub>650R</sub>			13.07 $\pm$ 1.49	S	S	100.0	0
B10.BR/SgSnJ	M	H-2 <sup>a</sup>	37.80 $\pm$ 10.44	S	S	66.1	100
B10.BR/SgSnJ <sub>650R</sub>			13.20 $\pm$ 2.62	S	S	66.1	0

\* All animals were infected with  $5 \times 10^3$  *T. b. r.* (LouTat-1).

† The survival times of the male and female C3HeB/FeJ unirradiated strain were separately compared with the survival times of the other intact male and female inbred strains by the Student's 2-tailed *t*-test. Similarly, the survival times of the male and female C3HeB/FeJ irradiated strain were separately compared with the survival times of the other irradiated male and female inbred strains. NS denotes that the values were not significant.

‡ Results are reported as significant (S) if the parasitemia for all the time points during the plateau period were different. NS denotes that none of the time points examined were statistically different from the C3HeB/FeJ animals at the 5% level.

ence was observed in their survival times in this study. In fact, it appears that the DBA/2J strain survived slightly longer than the AKR/J strain. The reason for this difference is unknown but could be due to the difference in the 2 clones of the EATRO 1886 strain that were used.

The differences in survival times between the susceptible C3H strains and the more resistant strains tended to become much smaller if the animals were given 650R of total body irradiation. However, data in Table I demonstrate that the irradiated C57 and the B10 strains survived significantly longer than the irradiated C3HeB/FeJ strain, suggesting that either a lower parasitemia will increase survival time, or that there

is a difference in their ability to adjust physiologically to the stress of the infection. None of the irradiated mouse strains showed any evidence of a drop in parasitemia or a relapse to another VAT. A comparison of the survival times of intact male with female mice of the resistant B10.A and B10.BR strains consistently showed that the female animals survived longer (Table II). This difference in resistance to the African trypanosomes between male and female mice has been previously observed (Greenblatt and Rosenstreich, 1984). It should be noted that no obvious sex differences were observed in reference to the other parameters examined. Both males and females of the various inbred strains ap-

TABLE III. *The kinetics of transformation of T. b. rhodesiense (LouTat-1) in 2 different irradiated inbred male mouse strains.\**

Inbred strain	Hours infected	Log <sub>10</sub> parasitemia $\pm$ SD	% Long slender $\pm$ SD	<i>P</i> † (%)	% Dividing $\pm$ SD	<i>P</i> (%)
C3HeB/FeJ <sub>650R</sub>	79	6.763 $\pm$ 0.349	100 $\pm$ 0		38.0 $\pm$ 6.56	
B10.BR/SgSnJ <sub>650R</sub>		7.000 $\pm$ 0.293	98.12 $\pm$ 1.78	NS	44.0 $\pm$ 11.56	NS
C3HeB/FeJ <sub>650R</sub>	102	8.065 $\pm$ 0.259	98.7 $\pm$ 1.71		29.44 $\pm$ 6.37	
B10.BR/SgSnJ <sub>650R</sub>		8.266 $\pm$ 0.174	86.3 $\pm$ 6.98	<0.1	15.72 $\pm$ 5.12	<0.1
C3HeB/FeJ <sub>650R</sub>	123	8.547 $\pm$ 0.193	80.95 $\pm$ 9.59		15.80 $\pm$ 5.00	
B10.BR/SgSnJ <sub>650R</sub>		8.566 $\pm$ 0.103	32.92 $\pm$ 8.80	<0.1	7.24 $\pm$ 3.47	<0.1
C3HeB/FeJ <sub>650R</sub>	152	8.741 $\pm$ 0.127	32.21 $\pm$ 7.98		6.41 $\pm$ 2.31	
B10.BR/SgSnJ <sub>650R</sub>		8.622 $\pm$ 0.060	22.23 $\pm$ 3.80	<1.0	4.65 $\pm$ 1.53	NS
C3HeB/FeJ <sub>650R</sub>	176	8.815 $\pm$ 0.088	24.96 $\pm$ 8.34		4.67 $\pm$ 1.99	
B10.BR/SgSnJ <sub>650R</sub>		8.562 $\pm$ 0.126	32.33 $\pm$ 6.15	<2.0	7.38 $\pm$ 1.77	<1.0

\* All animals infected with *T. b. r.* (LouTat-1) intraperitoneally at  $5.0 \times 10^3$  BT's.

† *P* (%), probability based on Student's 2-tailed *t*-test; NS, *P* > 5%. A comparison is made of the differences between the C3HeB/FeJ<sub>650R</sub> mice and the B10.BR/SgSnJ<sub>650R</sub> mice.

TABLE IV. The kinetics of transformation of *T. b. rhodesiense* (LouTat-1) in 3 different irradiated inbred female mouse strains.\*

Inbred strain	Hours infected	Log parasitemia $\pm$ SD	% Long slender $\pm$ SD	P† (%)	% Dividing $\pm$ SD	P (%)
C3HeB/FeJ <sub>650R</sub>	88.5	8.369 $\pm$ 0.081	98.50 $\pm$ 1.26		37.64 $\pm$ 4.42	
B10.A/SgSnJ <sub>650R</sub>		7.762 $\pm$ 0.235	99.76 $\pm$ 0.33		32.78 $\pm$ 3.35	
B10.BR/SgSnJ <sub>650R</sub>		7.953 $\pm$ 0.125	99.42 $\pm$ 0.58	NS	35.04 $\pm$ 1.06	NS
C3HeB/FeJ <sub>650R</sub>	112.5	8.956 $\pm$ 0.046	64.54 $\pm$ 9.53		12.82 $\pm$ 1.96	
B10.A/SgSnJ <sub>650R</sub>		8.637 $\pm$ 0.190	50.40 $\pm$ 23.00		12.50 $\pm$ 6.74	
B10.BR/SgSnJ <sub>650R</sub>		8.732 $\pm$ 0.081	32.40 $\pm$ 12.03	<0.2	6.94 $\pm$ 2.59	<1.0
C3HeB/FeJ <sub>650R</sub>	139.5	9.079 $\pm$ 0.083	33.22 $\pm$ 7.59		10.18 $\pm$ 3.23	
B10.A/SgSnJ <sub>650R</sub>		8.770 $\pm$ 0.106	23.74 $\pm$ 3.51		6.42 $\pm$ 1.74	
B10.BR/SgSnJ <sub>650R</sub>		8.772 $\pm$ 0.063	19.94 $\pm$ 3.66	<1.0	4.10 $\pm$ 1.55	<1.0
C3HeB/FeJ <sub>650R</sub>	162.0	9.325 $\pm$ 0.055	29.58 $\pm$ 4.11		6.98 $\pm$ 2.13	
B10.A/SgSnJ <sub>650R</sub>		8.875 $\pm$ 0.071	27.28 $\pm$ 3.37		8.62 $\pm$ 2.70	
B10.BR/SgSnJ <sub>650R</sub>		8.854 $\pm$ 0.060	27.66 $\pm$ 7.73	NS	5.48 $\pm$ 1.55	NS

\* All animals infected with *T. b. rh.* (LouTat-1) intraperitoneally at  $5.0 \times 10^3$  BT's.

† P (%), probability based on Student's 2-tailed *t*-test; NS, *P* > 5%. A comparison is made of the differences between the C3HeB/FeJ<sub>650R</sub> mice and the B10.BR/SgSnJ<sub>650R</sub> mice.

peared to have similar parasitemia levels during the plateau phase, and similar rates of morphological transition from the LS to the SS forms. In addition, in those strains that responded to the infection, there were no obvious differences found in the kinetics of clearance of parasitemia.

The early growth kinetics in both the irradiated and intact inbred strains were generally similar in that the trypanosome population eventually reached a plateau, and the transformation of the LS to the intermediate and the SS forms occurred at a similar rate (Fig. 1). There was, however, a statistically significant difference in the parasitemia level during the plateau phase between several of these inbred strains and the C3HeB/FeJ strain. For example, male and female C3HeB/FeJ mice consistently demonstrated a higher parasitemia during the plateau phase than either the B10.A or the B10.BR strains (Fig. 1; Tables I, II, also Tables III, IV). Similarly, the C3HeB/FeJ mice also had a statistically significant higher parasitemia during the plateau of parasitemia than the AKR/J and C57Bl/6By mice (Table I). It is of interest that female offspring of the F<sub>1</sub> cross between the C3HeB/FeJ and the B10.BR strains demonstrated a parasitemia that was initially similar to the C3H parent but later in the infection intermediate between the 2 parental strains (Fig. 2). The ability to control parasitemia therefore appears to be characteristic for each inbred strain of mice.

It is suggested that the differences in the height of the plateau in parasitemia of the different inbred strains is due to the rate at which the dividing LS population transforms to the non-

dividing SS population. The results show that the rate at which transformation occurred differed between the C3HeB/FeJ and B10 irradiated inbred strains (Tables III, IV). For example, the more resistant male B10.BR strain had a significantly lower percentage of LS trypanosomes at 102, 123, and 152 hr of infection than the

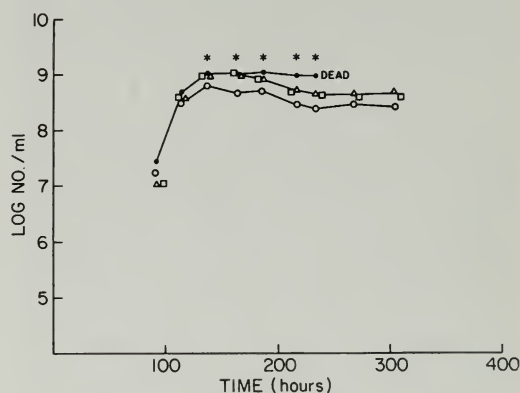


FIGURE 2. A comparison of the parasitemia in C3HeB/FeJ (●), B10.BR (○), and their F<sub>1</sub> cross (C3H × B10.BR) female irradiated mice (Δ, □)—the F<sub>1</sub> hybrid C3H female × the B10.BR male cross (Δ); and the F<sub>1</sub> hybrid C3H male and the B10.BR female cross (□). The \* indicates time points at which the parasitemias in irradiated C3H mice were significantly higher (5% level) than the irradiated B10.BR mice. At the first 2 time points (137.5 and 163.0 hr) during the plateau phase, the parasitemias of the F<sub>1</sub> mice were not statistically different from the C3H mice. At later timepoints, the F<sub>1</sub> parasitemias were statistically different from both parental lines.



TABLE V. The kinetics of transformation of *T. b. rhodesiense* (LouTat-1) in 3 different irradiated inbred male mouse strains.\*

Inbred strain	Hours infected	Log parasitemia $\pm$ SD	% Long slender $\pm$ SD	$P$ † (%)	% Dividing $\pm$ SD	$P$ (%)
C3HeB/FeJ <sub>650R</sub>	86	5.267 $\pm$ 0.363	99.8 $\pm$ 0.60		46.0 $\pm$ 9.9	
AKR/J		5.191 $\pm$ 0.331	97.5 $\pm$ 2.70		36.3 $\pm$ 13.5	
DBA/2J		5.816 $\pm$ 0.250	99.5 $\pm$ 0.71		43.4 $\pm$ 3.61	
C3HeB/FeJ <sub>650R</sub>	133	8.475 $\pm$ 0.153	74.7 $\pm$ 12.40	—	15.8 $\pm$ 6.3	—
AKR/J		8.191 $\pm$ 0.103	49.6 $\pm$ 16.20	<2.0	14.9 $\pm$ 3.0	NS
DBA/2J		8.526 $\pm$ 0.069	72.4 $\pm$ 10.04	NS	22.1 $\pm$ 4.1	NS
C3HeB/FeJ <sub>650R</sub>	146.5	8.812 $\pm$ 0.105	38.00 $\pm$ 0.04	—	9.37 $\pm$ 4.89	—
AKR/J		8.607 $\pm$ 0.203	23.26 $\pm$ 6.20	<1.0	7.33 $\pm$ 2.75	NS
DBA/2J		8.821 $\pm$ 0.035	48.80 $\pm$ 9.60	NS	14.00 $\pm$ 2.7	NS
C3HeB/FeJ <sub>650R</sub>	167	8.805 $\pm$ 0.089	25.9 $\pm$ 9.3	—	8.2 $\pm$ 3.6	—
AKR/J		8.540 $\pm$ 0.088	31.4 $\pm$ 12.9	NS	8.7 $\pm$ 1.3	NS
DBA/2J		8.557 $\pm$ 0.064	46.2 $\pm$ 1.6	<2.0	10.5 $\pm$ 0.5	NS
C3HeB/FeJ <sub>650R</sub>	190	8.615 $\pm$ 0.154	19.4 $\pm$ 12.2	—	6.0 $\pm$ 3.8	—
AKR/J		8.463 $\pm$ 0.157	29.7 $\pm$ 2.9	NS	10.0 $\pm$ 0.01	NS
DBA/2J		8.298 $\pm$ 0.077	39.7 $\pm$ 17.7	NS	13.6 $\pm$ 7.9	NS
C3HeB/FeJ <sub>650R</sub>	218	8.767 $\pm$ 0.147	28.6 $\pm$ 9.1	—	5.72 $\pm$ 2.5	—
AKR/J		8.452 $\pm$ 0.187	23.6 $\pm$ 8.5	NS	5.30 $\pm$ 1.5	NS
DBA/2J		8.586 $\pm$ 0.059	54.7 $\pm$ 18.5	<1.0	13.80 $\pm$ 5.5	<1.0

\* All animals infected with *T. b. r.* (LouTat-1) intraperitoneally at  $5.0 \times 10^3$  BT's. The results are a composite of 2 separate experimental runs.

†  $P$  (%), probability based on Student's 2-tailed  $t$ -test; NS,  $P > 5\%$ . A comparison is made of the differences between the C3HeB/FeJ<sub>650R</sub> mice and the AKR/J<sub>650R</sub> or DBA/2J<sub>650R</sub> mice.

irradiated male C3HeB/FeJ mice (Table III). In separate experiments similar results can be observed in Table V for the DBA and the AKR inbred strains. Again the results show that the AKR strain, which had a lower plateau in parasitemia than the C3H mice, also had a significant decrease in the percentage of LS dividing forms earlier in the infection. This is an important observation, because there are 2 possible mechanisms to explain the differences in the height of the parasitemia between 2 inbred mouse strains. One possibility could be that LouTat-1 has different growth rates in different inbred mice. The second could be that the growth rates of LouTat-1 are similar but that there are differences in the rate at which the morphological transformation occurs to the nondividing intermediate and SS stages. Because the growth kinetics did not appear to differ significantly in different inbred strains (either intact or irradiated), but differences were observed in the transition time, the latter explanation would appear to be the most likely one. Therefore, inbred strains of mice appear to have characteristic differences in their ability to induce the morphological transition of the LS stage to the SS stage, rather than in their ability to control the rate of trypanosome growth.

It can be noted in Table I that, in general, animal strains with the longer survival times had

the higher parasitemia indexes (PI), cleared their parasitemia in a majority of animals, and thus were capable of mounting a specific immune response. It is also obvious that the correlation between PI and survival time is not absolute. For example, the AKR/J strain, which had a PI of 82.2%, failed to produce a decrease in parasitemia, whereas the DBA/2J with a parasitemia and a PI that did not differ from the C3HeB/FeJ strain did so. This suggests that the innate ability of an animal strain to control the initial parasitemia cannot be solely responsible for resistance but is presumably only one of several factors. Further evidence that multiple factors are involved in determining survival times can be seen by comparing the survival times of the DBA/2J strain with the B10 strains. All 3 strains were capable of clearing their initial parasitemia and were, therefore, capable of producing an immune response to the initial parasitemia, yet both the B10 strains had significantly longer survival times than the DBA/2J strain (Table I). This would suggest a disassociation between the immune response and survival times. In addition, the female  $F_1$  offspring of the cross between the C3HeB/FeJ and the B10.BR parents demonstrated that 100% of the animals responded by clearing their initial parasitemia, regardless of the greater height of their initial parasitemia. Interestingly, although all the  $F_1$  animals cleared their parasit-

emia, their survival times more closely resembled that of the C3HeB/FeJ strain than the B10.BR strain.

Table I also shows that all the susceptible strains tested were capable of producing a relapse if the parasitemias were experimentally lowered. When the intact C3HeB/FeJ and AKR/J strains were infected and treated early with DFMO in order to maintain a parasitemia lower or similar to the B10 inbred strains, 100% of the animals cleared their parasitemia. However, if the parasitemias were allowed to approximate those observed in C3H mice infected with LouTat-1 prior to DFMO treatment, only 50% of the infected animals cleared their parasitemia and relapsed (Fig. 3). It should be noted that this clearance is immunologically induced, because a drop in parasitemia is not observed in DFMO-treated irradiated 650R or cyclophosphamide-treated animals. DFMO would appear to be trypanostatic, not trypanocidal. This latter observation on the effects of DFMO is identical to that reported by others (DeGee et al., 1983). To our knowledge, this is the first experimental demonstration to show that if parasitemias are lowered during an actual infection, then susceptible or normally nonresponding inbred strains are actually capable of clearing their parasitemia and mimicking the response of the resistant inbred strains.

### DISCUSSION

In work previously reviewed by Murray et al. (1982) and more recently discussed by Black et al. (1985) it was suggested that the resistant and susceptible phenotypes of different animal strains and species could be due to their ability to control the growth and morphological transition of the African trypanosomes nonspecifically. These investigators compared the C57Bl/6 intact inbred strain with the susceptible C3H/He strain when infected with *T. b. brucei*. However, to our knowledge, no one has systematically examined a series of inbred strains for their ability to control the initial parasitemia. Nor has there been a systematic examination of the hypothesis presented by these authors. The work reported here demonstrated that different inbred strains do show characteristic differences in their ability to control their parasitemia. It also demonstrated that this control is not due to a specific immune response. Finally, the results showed that the differences in the height of the parasitemia were due to the rate of transformation from the LS to the SS form. Previously, Balber (1972), using

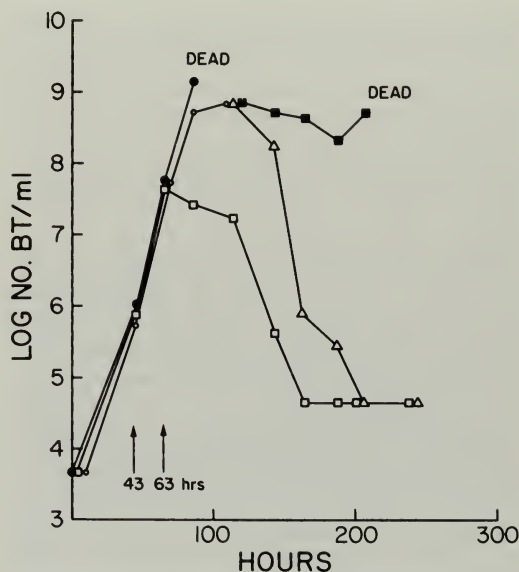


FIGURE 3. The response of C3HeB/FeJ mice to *T. b. g.* (TXtat-1) when treated with 2% DFMO in their drinking water. Control animals that received no treatment (●); animals that received 2% DFMO at 43–45 hr of infection (□) as shown by the first arrow. At this time trypanosomes were just detectable in their tail blood. Animals received 2% DFMO at 60–65 hr (○) as shown by the second arrow. Approximately 50% of the animals maintained a plateau in their parasitemia at an approximate average of log 8.5/ml until their death at greater than 200 hr (■), whereas the other 50% showed a definite decrease in parasitemia to numbers below log 5.0/ml (△). An almost identical set of data was obtained with the AKR/J animals.

irradiated male BALB/J mice, and Luckins (1972), using irradiated male albino Wistar rats demonstrated that the control of parasitemia was primarily due to nonspecific factors that regulated the rate of transition from the LS to the SS forms. Three mouse strains (C57, B10.A, and B10.BR) examined in this study that induced an earlier transformation showed greater resistance and were better able to mount an antibody response. From this data, it could be predicted that if the rate of transformation in some susceptible strains could be increased and the plateau in parasitemia lowered, animal strains that originally failed to clear their parasitemia would be able to do so. It has been clearly shown in both the C3HeB/FeJ and the AKR/J mice, which failed to clear their initial parasitemias, that if the parasitemia peaked at low enough numbers following treatment with DFMO, then all animals did clear their parasitemia. DFMO has been shown to induce a morphological change in the LS forms



that resembles the SS stage (DeGee et al., 1984), is trypanostatic, and in the absence of an intact immune system does not clear the animal of parasites (DeGee et al., 1983). The data just discussed are consistent with our previous predictions and would tend to strengthen the suggestion that the initial inherent control of parasitemia plays a role in resistance.

However, it is also clear from our results that this innate ability to induce the morphological change from the LS to the SS stage and to control the height of the parasitemia is not the only factor determining a mouse's ability to respond to the initial parasitemia. The AKR/J strain was able to control the initial parasitemia to almost the same degree as the resistant B10.BR strain, yet failed to produce a decrease in parasitemia in a majority of the animals. It had a survival time only slightly longer than the C3HeB/FeJ strain. Similarly, the data from the  $F_1$  cross between the C3HeB/FeJ and B10.BR parents demonstrated that the  $F_1$  hybrid had a higher parasitemia than the B10.BR strain yet 100% of the animals responded with antibody and cleared their parasitemia.

This suggests that the hypothesis that the innate factors that control the height of the parasitemia are responsible for the differences in the survival times of different inbred mouse strains is incorrect, or more probably, incomplete. Finally, it should be noted that all of the DBA/2J mice showed a decrease in parasitemia followed by a relapse to a new VAT similar to the more resistant B10 and C57 strains, yet failed to have survival times similar to these resistant strains. Therefore, survival times and the ability to respond immunologically to the initial parasitemia are not necessarily correlated. The disassociation between the immune response and survival time has been previously noted by DeGee and Mansfield in an elegant series of experiments (DeGee and Mansfield, 1984). The results from the  $F_1$  hybrid animals further support the statement that survival times and the ability to respond with antibody to the initial parasitemia are not correlated. The  $F_1$  hybrids all cleared their parasitemia but had survival times more similar to the susceptible C3H strains.

The inability to demonstrate a relationship between the ability of an inbred mouse strain to nonspecifically control its parasitemia, or its ability to induce an antibody response to the initial variant antigen population and its survival time are important observations and run counter

to current dogma. The results suggest that factors other than the specific immune response are important in resistance. It has been shown that animals infected with the African trypanosomes undergo profound physiological changes (Tizard, et al., 1978; Seed, 1980). It is therefore conceivable that the sensitivity to trypanosome catabolites, or the rate at which the pathological changes occur in different hosts is also important in determining the survival time of a particular host.

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## CROSS-RESISTANCE BETWEEN *SCHISTOSOMA MANSONI* AND *FASCIOLA HEPATICA* IN SHEEP

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**ABSTRACT:** Five sheep were exposed to 5,000 *S. mansoni* cercariae percutaneously and the stools examined for 20 wk to determine patency. The sheep were found to be partially susceptible to a primary infection and showed great individual variations in their pathophysiological responses. All of the sheep acquired a patent infection with *S. mansoni* and eggs were first seen in feces 9 wk postexposure with no eggs detected after 14 wk. At necropsy 20 wk postexposure only dead *S. mansoni* worms were found. KOH digests revealed that tissue egg counts were low, ranging from 0 to 133 in the liver, and 0 to 257 in the intestine. Primary infection of sheep with *S. mansoni* followed by oral infection with *F. hepatica* metacercariae 10 wk later resulted in a reduction of 51% in *F. hepatica* worms recovered over controls infected with *F. hepatica* for 10 wk. All 5 of the *S. mansoni*-infected/*F. hepatica*-challenged sheep developed 71 or less *F. hepatica* worms. In contrast, 3 of the 5 *F. hepatica*-infected sheep developed 113-197 worms. However, although the experimental mean worm burden was lower than the control group, the variability in the control group was too great to obtain significance between the groups. There was a clear tendency toward normocytic normochromic anemia following a primary infection with *S. mansoni*; however, blood values were more reduced in the *F. hepatica* challenge controls than in the animals that received primary infection with *S. mansoni*.

Investigators have shown that cross-resistance exists between *Fasciola* and *Schistosoma* species in several hosts. Hillyer (1976) and Christensen et al. (1978, 1980) demonstrated that adult *F. hepatica* infections induce significant resistance to subsequent challenge with *S. mansoni* in mice. Similarly, mice with mature primary infections with *S. mansoni* were found to show significant resistance to challenge with *F. hepatica* (Christensen et al., 1978; Hillyer, 1981). Christensen et al. (1980) also showed that mice with immature infections with *F. hepatica* develop resistance to challenge with *S. mansoni*.

Cross-resistance between schistosomes and liver flukes has also been demonstrated in farm animals. Monrad et al. (1981) found significant resistance to *F. hepatica* in sheep harboring primary infection with *S. bovis*. Heterologous resistance between *S. bovis* and *F. hepatica* or *F. gigantica* has also been demonstrated in cattle (Sirag et al., 1981; Yagi et al., 1985).

The objectives of the present study were to (1) evaluate the course of a primary infection of *S. mansoni* in sheep and (2) determine whether sheep sensitized with *S. mansoni* for 10 wk developed resistance to challenge infection with *F. hepatica*.

### MATERIALS AND METHODS

#### Experimental animals

Ten 3-mo-old lambs were purchased from a local farm that had no history of fascioliasis. Fecal samples from the lambs (as well as from other cattle in the farm) were done and no fluke or schistosome eggs were found. The lambs were transferred to the animal facilities at the UPR Medical Sciences Campus and maintained on concentrates with free access to water. *Monezia expansa* eggs were found in the feces of some lambs and thiabendazole was given for treatment before the experiment commenced.

#### Infective material

Cercariae of *S. mansoni* (Puerto Rican strain) were shed from *Biomphalaria glabrata* snails and used within 1 hr of shedding. Infection was carried out percutaneously with cercariae via the tail skin. A shaved area of the tail was situated in a plastic bag containing the cercariae suspended in dechlorinated tap water for 30 min. After exposure the cercarial fluid in plastic bags was checked and found to contain cercarial tails only.

Metacercariae of *F. hepatica* were purchased from Baldwin Aquatics, Inc. (Monmouth, Oregon). The presence of the characteristic ring-form appearance indicated the viability of cysts. For infection, they were counted, wrapped in a Whatman filter paper as a small bolus, and orally administered.

#### Fecal egg counts

The modified Ritchie formol-ether concentration technique described in Knight et al. (1976) was used for the detection and quantitation of *S. mansoni* and *F. hepatica* eggs.

#### Collection of blood and serum

Every 2 wk, lambs were bled from the jugular vein using vacutainer tubes containing ethylenediamine-

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tetraacetate (EDTA). For obtaining serum, blood was collected into vacutainers containing no anticoagulant. The blood was allowed to stand for several hours and then centrifuged at 2,000 *g* for 10 min. Serum was then separated and stored at -20 C until used.

### Hematological methods

Packed cell volume (PCV) was determined in a microhematocrit centrifuge (Clay Adams, Parsippany, New Jersey) and hemoglobin concentration (Hb) in a hemoglobinometer (American Optical Corporation, Buffalo, New York). Total white (WBC) and red (RBC) cell counts were performed on an electronic cell counter (Coulter Electronic ZF, Hialeah, Florida), and differential leucocyte counts were made on thin blood films stained with Wright's stain using the battlement technique (Schalm, 1965). Mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were calculated from Hb, RBC, and PVC values.

### Sorbitol dehydrogenase assay (SDH)

Serum sorbitol dehydrogenase (EC 1.1.1.14) was assayed using a commercial kit (Sigma Chemical Company, Saint Louis, Missouri) as an indicator of hepatic damage. It is more abundant in the liver than in any other organ and has been found in serum in cases of liver damage due to fascioliasis (Thorpe and Ford, 1969).

### ELISA

The ELISA was done exactly as described in Hillyer (1985). The polyvinyl plates were coated with either FhWWE or Fh<sub>SmIII(M)</sub>, both at a concentration of 4 µg/ml. The ovine sera were tested at a 1:16 dilution. The conjugate, used at a 1:1,000 dilution was a peroxidase-labeled affinity-purified antibody to (H + L) ovine IgG prepared in goats (Kierkegaard and Perry Laboratories, Gaithersburg, Maryland).

### Experimental design for infections

Ten lambs were divided into 2 equal groups of 5 (A and B). Each lamb in group A was infected percutaneously through a shaved tail with 5,000 cercariae of *S. mansoni*. Ten weeks later, each lamb in group A was infected with 400 metacercariae of *F. hepatica* together with 5 control lambs comprising group B. Blood and serum were collected every 2 wk and the following parameters were determined: complete hemogram, sorbitol dehydrogenase level in serum, and ELISA. Necropsy was carried out 10 wk after challenge with *F. hepatica* (20 wk after infection with *S. mansoni*), and the fluke burden in the livers was determined.

### Recovery of *S. mansoni* adult worms and eggs

At 20 wk postexposure to *S. mansoni* cercariae each lamb was kept off feed the night before necropsy to ensure an empty stomach and thus facilitate the perfusion process. One hour before necropsy the lamb was injected with 1 g of sodium antimony meso-2,3 dimercapto-succinate (Astiban, Hoffman La Roche, Basle) to induce a hepatic shift of worms. To prevent clotting, each lamb was injected intravenously with 1,500 i.u. of heparin 30 min before necropsy. The animal was then killed with an intravenous euthatol in-

TABLE I. Hematological data.

Group	Weeks*	PCV† %	Hb g/100 ml	RBC 10 <sup>6</sup> / cm <sup>2</sup>	TWBC 10 <sup>3</sup> /cm <sup>2</sup>	Eosino- phils 10 <sup>3</sup> /cm <sup>2</sup>
A	0	32.0	11.4	5.30	9,696	66
	2	36.8	12.1	8.33	11,008	503
	4	34.6	12.7	9.86	14,699	1,084
	6	33.0	11.7	8.29	17,028	1,496
	8	31.8	11.5	8.04	19,703	2,579
	10 (0)	32.8	11.1	7.86	23,181	2,880
	12 (2)	32.2	12.0	6.57	19,983	3,704
	14 (4)	33.8	12.0	6.24	15,529	5,279
	16 (6)	32.6	11.5	7.32	24,087	6,021
	18 (8)	30.8	11.4	6.32	22,515	3,348
	20 (10)	31.0	11.4	6.37	15,116	3,720
B	(0)	30.0	10.8	6.79	9,839	117
	(2)	34.4	12.6	7.21	16,025	1,648
	(4)	30.6	11.7	6.29	12,524	1,905
	(6)	30.6	11.1	6.76	17,599	3,087
	(8)	29.4	10.6	5.57	14,270	2,635
	(10)	29.2	11.2	5.71	12,551	3,041

\* 0-20 = weeks of *S. mansoni* infection. Group A mixed *S. mansoni* + challenge *F. hepatica* (weeks 10-20). (0)-(10) = weeks. *F. hepatica* infection.

† For abbreviations see Materials and Methods.

jection; the posterior vena cava and the thoracic aorta were ligated, and perfusion was carried out through an incision in the thoracic aorta posterior to the ligated site with a citrated normal saline from a container situated 1.5 m above the carcass by means of a pump. The portal vein was connected by plastic tubing to a large container in which the perfusate containing the worms was collected. Each perfusion took at least 2 hr.

Tissue *S. mansoni* egg counts were made by the KOH digestion method of Cheever (1970) using representative pieces from different sites of the liver and intestine. Digestion was carried out overnight, in 4% KOH at 37 C.

### Recovery of *F. hepatica* adult worms

Adult flukes were first collected from the bile ducts, then the liver was sliced into small pieces about 1 cm<sup>2</sup> and kept in normal saline for 2 hr. Each piece was then pressed and the flukes that emerged were collected. Only complete worms or fluke heads were counted.

## RESULTS

### Fecal egg counts of *S. mansoni*

Two of the 5 sheep infected with *S. mansoni* had 2-5 eggs/g (epg) at 9 wk postexposure. All 5 had 1-9 epg by 10-11 wk of infection but were again negative for *S. mansoni* eggs by 14 wk postexposure and afterwards through the 20 wk tested. Fecal egg counts of *S. mansoni* were always low, ranging from 1 to 9 epg.

### Tissue egg counts of *S. mansoni*

At necropsy, 20 wk postexposure to *S. mansoni* the amounts of eggs found in the livers and intestines were estimated following digestion with



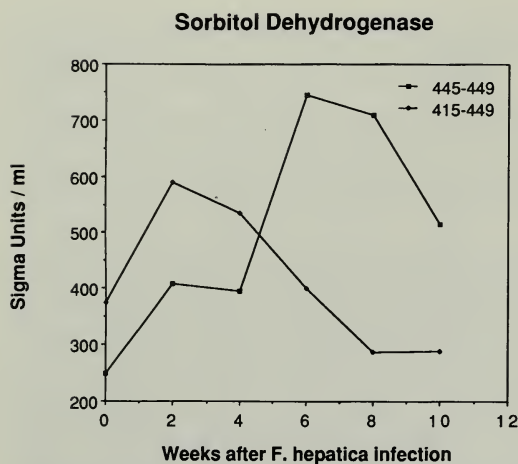


FIGURE 1. Serum sorbitol dehydrogenase levels of sheep primarily infected with *Schistosoma mansoni* for 10 wk (week 0) and challenged with *Fasciola hepatica* for an additional 10 wk (415-419) and *F. hepatica*-infected challenge controls (445-449).

KOH. Two of the 5 sheep did not have any detectable eggs, and in these only 1 egg was found in the feces during 10-13 wk of infection with *S. mansoni*. The remaining 3 had 121, 127, and 133 epg in the liver and 181, 219, and 257 epg in their intestines. No *F. hepatica* eggs were found in the KOH digest.

#### Hematological results

The hematological findings are summarized in Table I. Infection with *S. mansoni* resulted in an increase in total leukocyte counts and eosinophils peaking at 8-10 wk. Regarding the doubly infected (*S. mansoni*-infected/*F. hepatica*-challenged) group versus the *F. hepatica* challenge controls the only major differences were as follows: total leukocyte counts were higher in the doubly infected group than those of the *F. hepatica* challenge controls throughout the experiment but significant differences between the counts of the 2 groups were only seen at the eighth week of the challenge infection. Likewise, there were also higher eosinophil counts in the *S. mansoni*-infected/*F. hepatica* group than the *F. hepatica* challenge controls but the only significant difference was at the sixth week of the challenge infection.

#### Sorbitol dehydrogenase assay

There were higher sorbitol dehydrogenase values in the *F. hepatica* challenge controls than in the *S. mansoni*-infected/*F. hepatica*-challenged group of lambs from the sixth week of challenge

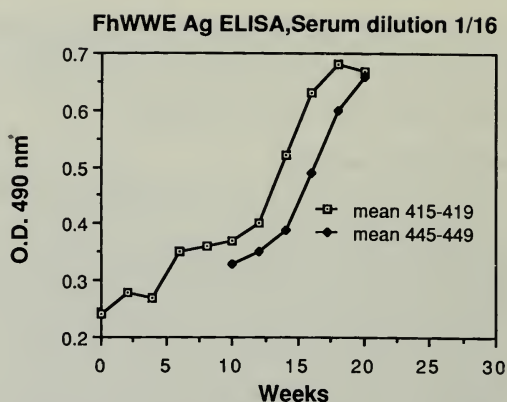


FIGURE 2. Mean ELISA absorbance values of sheep infected with *Schistosoma mansoni* for 0-20 wk and challenged with *Fasciola hepatica* at week 10 (415-419), and challenge controls infected with *F. hepatica* for 0-10 wk (weeks 10-20) (445-449) and reacted against a crude *F. hepatica* worm extract.

until the end of the experiment with significant differences at the sixth and eighth week of challenge (Fig. 1).

#### Fluke recovery

There was a reduction of 51% in the number of *F. hepatica* flukes recovered from group A as compared with the worm recovery from the challenge controls (Table II). It should be noted, however, that the fluke recoveries from 3 challenge control lambs were 113, 132, and 197 compared to a maximum fluke recovery of 71 from the *S. mansoni*-infected/*F. hepatica*-challenged lambs. Taken as a whole, however, this reduction was statistically insignificant. No *S. mansoni* worms were found in the sheep infected for 20 wk.

#### ELISA

As detected by ELISA, antibody levels to crude *Fasciola* antigens rose gradually through 12 wk of infection with *S. mansoni* (and 2 with *F. hepatica*) and then rose rapidly through 8 wk of *F. hepatica* (18 with *S. mansoni*) infection in parallel with the *F. hepatica*-infected controls (Fig. 2). With Fh<sub>SmIII(M)</sub> the antibody levels rose due to *S. mansoni* infection and then rose even higher due to *F. hepatica* infection; however, this last rise of antibody level was also concurrent with the increase seen in the *F. hepatica*-infected controls (Fig. 3).

#### DISCUSSION

A primary infection of *S. mansoni* in sheep resulted in a short-term patent *S. mansoni* in-

TABLE II. Recovery of *F. hepatica* adult worms.

Group	Sheep no.	No. of <i>F. hepatica</i> recovered	% Reduction	Mean $\pm$ SD
A*	415	27	51	51 $\pm$ 19
	416	71		
	417	47		
	418	43		
	419	69		
B†	445	50		104 $\pm$ 68
	446	27		
	447	132		
	448	113		
	449	197		

\* Five thousand cercariae of *S. mansoni* for 10 wk + 400 metacercariae/cercariae of *F. hepatica*.

† Four hundred metacercariae/cercariae of *F. hepatica*.

fection that was quickly eliminated by the host. Eggs were first seen at 9 wk, i.e., 3 wk after it is seen in the human host (Hillyer, 1982). Moreover, in spite of exposure to 5,000 cercariae the infections must have been invariably light as egg excretion levels were very low. Saeed and Nelson (1974) exposed sheep to 20,000 *S. mansoni* cercariae. One lamb killed on the 14th week of infection harbored 617 worms (3%). Eggs were first seen in feces at 16 wk of infection. With this high exposure, the last sheep was killed at 25 wk post-exposure and harbored 1,444 worms (7.5%) and 3 times the amount of epg in the liver found as in our present experiment. In contrast to Saeed and Nelson (1974), our sheep apparently self-cured as no *S. mansoni* worms were found at necropsy.

Our results demonstrated that sensitization with a primary *S. mansoni* infection of sheep followed 10 wk later with a challenge infection with *F. hepatica* resulted in a reduction of 51% in *F. hepatica* worms recovered as compared with unsensitized controls. Although this reduction was statistically insignificant, it should be noted that the fluke recovery from 3 challenge control lambs was 113, 132, and 197 compared to a maximum fluke recovery of 71 from the previously infected lambs. Similar results were found by Damian et al. (1984) when vaccinating baboons with cryopreserved schistosomules. Here, partial protection was found in only half of the vaccinated animals. Both in their study as well as ours, outbred animals were used and this may explain in part the heterogeneous results obtained.

The sheep also displayed a tendency toward normocytic normochromic anemia in the challenge controls than the previously infected lambs.

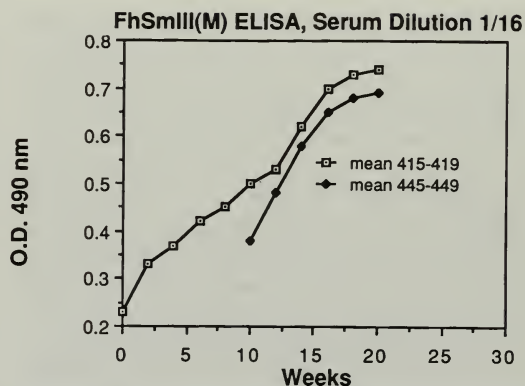


FIGURE 3. Mean ELISA absorbance values of sheep infected with *Schistosoma mansoni* for 0–20 wk and challenged with *Fasciola hepatica* at week 10 (415–419), and challenge controls infected with *F. hepatica* for 0–10 wk (weeks 10–20) (445–449) and reacted against a *Fasciola/Schistosoma* cross-reactive, cross-protective antigen denoted FhSmIII(M).

A similar type of anemia was reported in sheep infected with *F. hepatica* (Sinclair, 1962) and *F. gigantica* (Haroun et al., 1986), although Furmaga and Gundlach (1967) and Ross et al. (1967) encountered a macrocytic normochromic type. Normocytic normochromic anemia has also been reported in sheep experimentally infected with *S. bovis* (Saad, 1979). Hemorrhage has been found to be the cause of anemia in ovine fascioliasis (Dargie et al., 1970) in ovine schistosomiasis (Saad, 1979), as well as in bovine schistosomiasis (Saad et al., 1984).

Sorbitol dehydrogenase values also indicated more hepatic damage in the challenge (*F. hepatica*) controls than in the previously infected (*S. mansoni*) lambs. This enzyme has been reported to be a good indicator of hepatic damage due to *F. hepatica* (Sewell, 1967; Sinclair, 1973) or *F. gigantica* in sheep (El Samani et al., 1985; Haroun et al., 1986).

Collectively, these results suggest the development in sheep of partial resistance stimulated by primary infection with *S. mansoni* against challenge infection with *F. hepatica*.

The presence of shared antigens between the 2 trematodes was indicated by ELISA. There was an elevation of IgG antibody levels detected by FhWWE and FhSmIII(M) by the 10th week of *S. mansoni* infection (i.e., the time of challenge with *F. hepatica*).

Evidence for the involvement of immunological factors in the cross-resistance between schistosomes and liver flukes (rather than mere physical changes due to primary sensitizing infection)



was indicated by the success of crude or purified antigens of *F. hepatica* to stimulate resistance to challenge with *S. mansoni* in mice and hamsters (Hillyer, 1979, 1981; Hillyer and Serrano, 1982), as well as resistance to *F. hepatica* in mice (Hillyer, 1985) and cattle (Haroun and Hillyer, 1986). Furthermore, the protective *F. hepatica* worm antigens were those that bound to *S. mansoni* antibodies and that as antigen purification proceeded, smaller amounts were required to obtain significantly high levels of protection.

In the present experiment, sheep were found to be partially susceptible to *S. mansoni* and the fecal egg counts were low 10 wk after infection. Hence, the pathological changes per se would not be expected to constitute a physical barrier to the challenge infection with *F. hepatica*. Similar findings have been reported in sheep harboring primary nonpatent (2–3 wk old) and newly patent (7–8 wk old) *S. bovis* infections. Challenge infections with *F. hepatica* were reduced by 70% and 93%, respectively, however, no resistance was found in sheep harboring primary 16–17-wk-old *S. bovis* infections (Monrad et al., 1981). It was concluded that the presence of eggs in the tissues or mature eggs producing *S. bovis* at the time of challenge are not important for the initiation of cross-resistance in sheep. Thus, it is possible that some stage-specific, cross-reactive antigens of *S. bovis* or *S. mansoni* stimulate the resistance against *F. hepatica* in sheep.

#### ACKNOWLEDGMENTS

These investigations were supported by USPHS Grant No. R22 AI20974 administered by the U.S.-Japan Cooperative Medical Science Program. E.T.H. was a USPH-NIH Fogarty International Fellow on sabbatical leave from the Department of Pathology, Faculty of Veterinary Sciences, University of Khartoum, Sudan. The cost for maintenance of the sheep at the UPR Medical Sciences Campus was done thanks to a grant from the graduate studies program and is warmly acknowledged. The assistance of Sharon File-Emperador in several aspects of the study and especially in supervising the infections with *S. mansoni* cercariae is deeply appreciated.

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## BOOK REVIEW . . .

**Chemotherapy of Tropical Diseases, Volume 21, Clinical Reports on Applied Chemistry**, M. Hooper (ed.). John Wiley and Sons, Chichester, Great Britain. 1987. 104 p.

This book is Volume 21 of *Critical Reports on Applied Chemistry*, for which "the aim is to provide . . . an overview of the scientific . . . developments affecting the chemical and allied industries." The first chapter is an introductory one by L. E. Goodwin, who reviews chemotherapy of the six tropical diseases targeted by the WHO Special Programme in Tropical Diseases: malaria, trypanosomiasis, leishmaniasis, schistosomiasis, filariasis, and leprosy. Goodwin efficiently summarizes the diseases' threats, the advantages and deficiencies of classic drugs for these diseases, and the potential of newer agents. The summary of antimalarials is particularly up-to-date.

This first chapter leads the reader to expect that each of the six targeted diseases will be discussed in the following chapters. Instead, the rest of the book (chapters 2-4) considers *Mycobacteria*, *Filaria*, and *Trypanosomes/Leishmania*, respectively. This slim, 104-page volume would have been improved if an editorial decision had been made to add 50 pages devoted to the chemotherapy of malaria and schistosomiasis.

Chapter 2, by D. E. Minnikin, consists of a detailed review of the structure and synthesis of the cell envelope of *Mycobacterium leprae*, the etiologic agent of leprosy, and of the other *Mycobacteria*. Topics include the structure of the peptidoglycan arabinogalactan that is the innermost part of the cell envelope; the structure and biosynthesis of the very long chain mycolic acids that attach to the glycan; and the structure and biosynthesis of the free lipids (such as phthicerol dimycocerosates, trehalose dimycolates, and glycosylated peptidolipids) that are found in the outer regions of the mycobacterial cell envelope. The discussion of these complex biochemicals is aided by clear diagrams of chemical structures. This detailed presentation of one aspect of mycobacterial biochemistry is interesting. Nevertheless, except for cycloserine, present mycobacterial chemotherapy is not based on inhibition of cell envelope biosynthesis, and the chapter would have been aided if biochemical pathways related to actual as well as to potential therapy had been discussed.

The third chapter, by D. A. Denham and J. Barrett, concerns filarial chemotherapy. The chapter begins with a discussion of the therapeutic index of diethylcarbam-

azine, followed by a one-sentence statement about the "newly introduced microfilaricide, ivermectin." I could find no references more recent than 1984, and the lack of emphasis on ivermectin, which appears to be replacing diethylcarbamazine as an antifilarial, is a deficiency. The bulk of the chapter is a discussion of filarial carbohydrate, lipid, and amino acid metabolism; respiration; folate and nucleic acid metabolism; and transport. A section on potential chemotherapeutic targets emphasizes the importance of carbohydrate metabolism, which is appropriate to the time at which the chapter was written, but not to the present situation in which ivermectin and nematode neurotransmission are a focus of scientific investigation. A nice section follows in which screening methods for filaricides are evaluated. Too often, reviews of chemotherapy are non-critical in the sense that results from all screens are lumped together without informing the reader which screening models are the most reliable.

The fourth and final chapter, by J. B. Brown, concerns trypanosomiasis and leishmaniasis. The essence of this chapter is a well-written review of trypanosomid carbohydrate, purine, and polyamine metabolism. Lipid metabolism is underemphasized, unfortunately. A nice discussion of encapsulated drugs and of their potential concludes this chapter.

A few minor misspellings were found: the correct spelling of the new antimalarial (page 8) is ginghamosu (artemisinin). On page 90, structure (20) should contain a pyridine ring rather than a benzene ring.

This volume is useful if the reader wishes an introduction to those issues discussed: the biochemistry of filaria, trypanosomids, and the mycobacterial cell envelope up to approximately 1984. As befits a publication on applied chemistry, chemical diagrams are clear and frequent. The apparent date of completion of these articles and the decision to limit the subject matter to these four organisms precludes this volume from being generally useful to those interested in biochemical pharmacology of parasites of clinical significance in 1988. Nevertheless, the basic argument of all the authors—that parasite biochemistry is specific and offers exciting avenues of chemotherapeutic attack—is correct and adds to the volume's value.

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## GENETIC DIFFERENTIATION AND BIOCHEMICAL POLYMORPHISM AMONG TRICHOMONADS

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**ABSTRACT:** Isoenzyme electrophoresis was used to study levels of genetic differentiation among strains and clones of *Trichomonas gallinae*, *Trichomonas vaginalis*, *Tritrichomonas foetus*, *Tetratrichomonas gallinarum*, and *Pentatrichomonas hominis*. Strain variation was found within *T. gallinae*, *T. vaginalis*, and *T. foetus*, however, levels of enzyme polymorphism were greater in *T. gallinae* than in *T. vaginalis* or *T. foetus*. Isoenzyme genotypes were not a stable property of *T. gallinae* clones cultivated *in vitro*. Retrospective studies of *T. gallinae* SG and JB6 clones revealed that mutation occurred during *in vitro* cultivation. Heterozygotes of hexokinase-1 and phosphoglucumutase displayed 2 allomorphs in equal dosage, indicating that trichomonads are diploid for these protein loci. Phenetic clustering of the biochemical data suggests that levels of genetic divergence among the species studied are extensive.

Isoenzyme electrophoresis has proven to be a useful tool for studying protozoan genetics and systematics (Carter, 1978; Gibson et al., 1980; Kreutzer and Sousa, 1981; Chapman, 1982; Moss et al., 1986; O'Donoghue et al., 1986). Previous isoenzyme studies of trichomonads have focused on conspecific variation in isoenzyme phenotypes (Chyle et al., 1971; Takayanagi et al., 1971; Soliman et al., 1982; Coombs and North, 1983; Gradus and Matthews, 1985), although congeneric and intergeneric differences in the mobilities of certain proteins have been occasionally noted. Unfortunately, genetic interpretations of electrophoretic phenotypes and comparisons of different taxa have been lacking. In addition, investigators have not tested for correlations between isoenzyme phenotypes and virulence, geographic distribution, or antigenic properties of trichomonads.

In the present study, isoenzyme data are presented for *Trichomonas gallinae* (Rivolta), *Trichomonas vaginalis* Donné, *Tritrichomonas foetus* (Riedmüller), *Tetratrichomonas gallinarum* (Martin and Robertson), and *Pentatrichomonas hominis* (Davaine). Levels of biochemical polymorphism and genetic divergence among the above taxa are described and discussed.

### MATERIALS AND METHODS

#### Organisms

All trichomonads used in this investigation were grown from cryopreserved stablates. The following

genera, species, strains, and clones (cl) were used (geographic origin of stablate in parentheses): *T. gallinae* Jones' Barn (JB) strain isolate 6 (Pennsylvania, U.S.A.), JB6 cl(s) 1, 7, and 8; Stabler-gallinae (SG) cl 1 (Colorado, U.S.A.), SG scl 1 (a subclone of SG cl 1); SG cl 1 (derived from SG cl 1 by 12 mo of continuous *in vitro* cultivation); *T. vaginalis* TvK1 strain (isolated by Kupferberg and Trussell in 1939, U.S.A.), Balt strains 23, 41, 42, 44, 53, 55, 70, and 80 (Maryland, U.S.A.), Balt 42 cl(s) 4, 5, 6, 7, 8, 9, and 11, Balt 44, cl(s) 4, 5, and 7, JH 31A strain (Maryland, U.S.A.), JH 31A, cl(s) 7, 8, and 10; *T. foetus* KV (Karlov Vary, Czechoslovakia) cl 1 (KV-1), CB strain (Colorado, U.S.A.), UT (Utah, U.S.A.) cl 1 (UT-1), COLO cl 1 (Colorado, U.S.A.), and DK (California, U.S.A.) cl 2 (DK-2); *T. gallinarum* cl 1 (Massachusetts, U.S.A.); *P. hominis* cl 1 (Korea). Methods of isolation and histories of most of these strains and clones have been previously documented (Honigberg, 1961; Honigberg et al., 1966, 1970, 1984; Kulda, 1967; Honigberg and Goldman, 1968; Kulda and Honigberg, 1969; Stepkowski and Honigberg, 1972; Kulda et al., 1974; Su-Lin and Honigberg, 1983). The IBERG strain of *T. gallinae* was isolated on 5 May 1980 from throat swabs of a pigeon obtained from Dr. R. M. Stabler (Colorado). This strain was transferred twice in Diamond's TYM, pH 7.0, containing 1,000 U penicillin and 1,000 µg streptomycin/ml, transferred to TYM without antibiotics, and cryopreserved. Infections with the IBERG strain are usually fatal for pigeons. *Trichomonas gallinae* SG scl 1 was obtained by subcloning from SG cl 1 during the course of this investigation. An attenuated strain of *T. gallinae*, JB6 cl 8 att (attenuation confirmed by pigeon inoculation), was obtained by maintaining virulent JB6 cl 8 in culture for 144 days. *Tritrichomonas foetus* isolates CB strain and COLO cl 1 were both isolated from bulls in Colorado during 1967 and maintained as frozen stablates.

#### Cultivation

All *T. vaginalis* strains and clones were grown in Diamond's trypticase-yeast-maltose medium (TYM) (Diamond, 1957), pH 6.0, supplemented with 10% v/v heat-inactivated horse serum. Stablates were first cultivated in TYM and were subsequently transferred to TYM without agar. *Trichomonas gallinae*, *T. foetus*,

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TABLE I. *Enzymes resolved in the trichomonad taxa.*

Abbreviation	Enzyme	E.C. No.
ACP	Acid phosphatase	3.1.3.2
ALD	Fructose-biphosphate aldolase	4.1.2.13
G6PD	Glucose-6-phosphate dehydrogenase	1.1.1.49
HI	Phosphohexose isomerase	5.3.1.9
HK-1	Hexokinase-1	2.7.1.1
HK-2	Hexokinase-2	2.7.1.1
LDH	Lactate dehydrogenase	1.1.1.27
PEP-B	Leucyl-glycyl-glycine peptidase	3.4.11.13
MDH-1	Malate dehydrogenase-1	1.1.1.37
MDH-2	Malate dehydrogenase-2	1.1.1.37
PGM	Phosphoglucomutase	5.4.2.2
UAE	Umbelliferyl acetate esterase	3.1.1.1

*T. gallinarum*, and *P. hominis* were grown in cysteine-tryptose-liver-maltose medium (CTLM) (McEntegart, 1952), pH 7.3, or Diamond's TYM, pH 7.0 (modified by substituting BBL trypticase peptone for trypticase), media without agar, supplemented with 10% heat-inactivated horse serum. Log-phase trichomonads were harvested by centrifugation and subsequently washed 3 times in 10 volumes of an ice-cold homogenizing solution containing 6 g sucrose and 10 mg each of dithiothreitol,  $\beta$ -NAD, and  $\beta$ -NADP/100 ml of distilled water. Washed organisms were pelleted at 11,600 g and frozen at  $-70^{\circ}\text{C}$ . Frozen samples were used within 3 mo of the time of harvest.

### Electrophoresis

Immediately before use, cell pellets were thawed, suspended in 2 volumes of homogenizing solution, and disrupted using either a probe sonicator or a Dounce homogenizer. Homogenates were centrifuged at 11,600 g for 3 min to remove cell debris, and samples of the supernatant fluid were subjected to vertical starch gel electrophoresis at 4  $^{\circ}\text{C}$ . The following buffers were used for electrophoresis: (a) phosphate-citrate, pH 6.8 (60 mM  $\text{Na}_2\text{HPO}_4$ , 10 mM citric acid) diluted 1:4 for gel and undiluted for electrode bath; (b) Tris-maleate, pH 7.5 (100 mM Tris adjusted to pH 7.5 with 2 M maleic acid) diluted 1:3 for gel and undiluted for electrode bath.

### Enzyme localization

Following electrophoresis, proteins were visualized in gel slices by procedures described in Harris and Hopkinson (1976). Acid phosphatase was detected by the method of El-Sharkawy and Huisinsh (1971). See Table I for enzymes resolved, their abbreviations, and Enzyme Commission numbers. Control gel slices developed without the corresponding specific substrates (e.g., lactate dehydrogenase without sodium lactate), yielded no detectable stain reactions.

Differences in phenotypes at a specific locus were used to deduce genotypes of clones or the number of allomorphs occurring in noncloned strains. For each locus, the allomorph of fastest anodal mobility was designated A; then, the others in order of decreasing mobilities, as B, C, etc. When more than 1 presumed locus yielded products with the same enzymatic activity, the locus with isozymes of faster anodal mobility was designated 1 and that of slower mobility as 2.

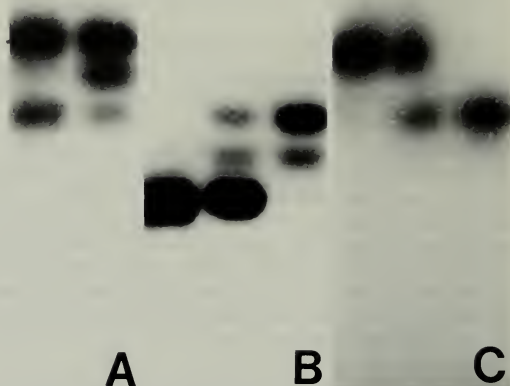


FIGURE 1. Phenotypes of some polymorphic trichomonad enzymes. The anode is at the top. Taxa and their presumed genotypes (in italics) are read left to right. Methods of allomorph designation are described in the Materials and Methods. A. Hexokinase loci (HK) 1 and 2. *Trichomonas gallinae* JB6 cl 7, HK-1 CC, HK-2 CC; *T. gallinae* JB6 cl 1, 1985 stabilate, HK-1 CD, HK-2 CC. The HK-1 heterozygote has 2 bands with a 1:1 staining intensity, indicating a monomeric subunit structure. B. Phosphoglucomutase (PGM). *T. gallinae* JB6 cl 7, PGM DD; *T. gallinae* JB6 cl 1, 1985 stabilate, PGM BCD; *T. gallinae* SGC cl 1, PGM BC. Heterozygote phenotype characteristic of a monomeric protein. C. Leucyl-glycyl-glycine peptidase (PEP-B). *Pentatrichomonas hominis* cl 1, PEP-B AA; *Trichomonas vaginalis* Balt 80, PEP-B AB; *T. gallinae* IBERG, PEP-B BB.

### Data analysis

The BIOSYS-1 computer program (Swofford and Selander, 1981) was used to analyze genetic data and produce the UPGMA phenogram. Clustering procedures were performed using only those 9 loci that were scored in all species (i.e., excluding the proteins acid phosphatase, lactate dehydrogenase, and umbelliferyl acetate esterase in Table II).

### RESULTS

Representative isoenzyme phenotypes and their corresponding genotypes are shown in Figure 1. Additional enzymes that showed activity on gel slices, but were not adequately resolved for interpretation included: glutamate-oxaloacetate transaminase, glutamate dehydrogenase,  $\alpha$ -naphthyl acetate esterase, superoxide dismutase, and catalase. In a few instances, a protein resolved in one species or strain lacked sufficient activity to be interpreted in another. In other cases, a protein with more than 1 locus in one species sometimes showed only a single activity zone in another. In such instances, the single

zone of activity was considered to be composed of 2 comigrating loci.

Malate dehydrogenase-1 and -2 were the only loci with more than 1 phenotype in *T. foetus*, and they served to distinguish KV-1 from the other strains and clones. Polymorphic loci in *T. gallinae* included: hexokinase-1, hexokinase-2, phosphoglucomutase, malate dehydrogenase-1, and malate dehydrogenase-2. Heterozygotes were observed for *T. gallinae* hexokinase-1 and phosphoglucomutase only (Fig. 1A, B). Polymorphic loci in *T. vaginalis* were restricted to phosphoglucomutase and leucyl-glycyl-glycine peptidase (Fig. 1C) in Balt 41 and Balt 80 strains, respectively. This was the only protein variation detected among the 23 stablates of this species examined in the present study. These stablates represented 9 strains, 13 clones obtained from these strains, and 1 strain (TvK1) that was isolated in 1939.

Studies of extant clones of *T. gallinae* JB6 indicate that they are not genetically identical (Table II). For example, JB6 cl 1 has a CD genotype for hexokinase-1, whereas JB6 clones 7 and 8 have a CC genotype at this locus. The parent strain of these clones, JB6, also has a CC hexokinase-1 genotype and is identical to clones 7 and 8 at all other loci. This suggests that either the D allomorph was lost from the parent strain subsequent to cloning, or that a new allomorph is being expressed in JB6 cl 1. In support of the second explanation, retrospective studies revealed that isolates of JB6 cl 1 and SG cl 1 have undergone genetic changes during *in vitro* cultivation. For example, a 1970 stablate of JB6 cl 1 yielded a homozygous DD phosphoglucomutase genotype, however, a 1985 stablate of the same clone yielded a BCD phosphoglucomutase phenotype with a complex gene dosage (Fig. 1B). In the case of SG cl 1, stablates of the original clone yielded a BCD phosphoglucomutase phenotype, indicating that this was not a successful clone, but instead a mixed population. This was substantiated by subcloning, which yielded SG scl 1 with a DD phosphoglucomutase genotype. In contrast to the situation with JB6 cl 1, prolonged cultivation of SG cl 1 (i.e., SGC cl 1) produced a normal dosage phosphoglucomutase BC genotype (Fig. 1B).

The number and relative staining intensity of isozyme bands in clones heterozygous for individual protein loci were used to deduce the subunit structures of those enzymes. Heterozygotes of hexokinase-1 and phosphoglucomutase dis-

played 2 bands of equal staining intensity, indicating that these proteins are monomers (Fig. 1A, B).

Some trichomonad strains can be conclusively diagnosed from their isoenzyme phenotypes (Table II). Among *T. gallinae* strains, the hexokinase-2 locus "C" allele distinguishes the virulent JB and IBERG strains from the avirulent SG strain. In addition, the highly virulent *T. foetus* KV-1 isolate can be distinguished from those clones and strains of intermediate and low virulence (DK-2, CB, UT-1, and COLO cl 1) by the malate dehydrogenase-1 and malate dehydrogenase-2 loci. In contrast, no differentiating allomorphs were detected among *T. vaginalis* Balt strains that differed in their inherent virulence as previously evaluated on the basis of the pathologic changes they caused in women, and by the subcutaneous mouse assay (Honigberg et al., 1984). Finally, a stablate of *T. gallinae* JB6 cl 8, which was virulent for pigeons, had an isoenzyme profile identical to a stablate of JB6 cl 8 that had been attenuated by 144 days of *in vitro* cultivation.

A matrix of Rogers' (1972) genetic similarity and Nei's (1972) genetic distance coefficients among representative taxa are presented in Table III. Pairwise comparisons of species yielded extremely low genetic similarity levels. Phenetic clustering (UPGMA) of the genetic data (Fig. 2) produced 3 major groups. *Pentatrichomonas hominis* showed the lowest level of genetic similarity to the other trichomonad taxa. The 2 urogenital species of mammals (*Trichomonas vaginalis* and *Tritrichomonas foetus*) and the 2 gastrointestinal species from birds (*Trichomonas gallinae* and *Tetratrichomonas gallinarum*) clustered as 2 distinct groups. Within *T. gallinae*, the 2 isolates from Colorado (IBERG and SG cl 1) showed low levels of genetic similarity in comparison to the isolate from Pennsylvania (JB6).

## DISCUSSION

Differences in levels of enzyme polymorphism were found between *Trichomonas gallinae* and *Trichomonas vaginalis*. Five of 11 loci surveyed were polymorphic in *T. gallinae*, but only 2 of 11 loci were polymorphic in *T. vaginalis*. Two polymorphic loci were detected in *Tritrichomonas foetus*, however, only 5 stablates representing 5 strains were surveyed. Alderete (1983) also reported homogeneity in the protein profiles of 5 strains of *T. vaginalis* analyzed by SDS-PAGE. The level of *T. vaginalis* enzyme poly-



TABLE II. *Electrophoretic genotypes in trichomonad taxa.\**

<i>Trichomonas gallinae</i>								
Locus	JB6	JB6 cl(s) 7, 8 JB6 cl 8 (att)	JB6 cl 1 (1970)	JB6 cl 1 (1985)	IBERG	SG cl 1	SG scl 1	SGC cl 1
ACP	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
ALD	DD	DD	DD	DD	DD	DD	DD	DD
G6PD	BB	BB	BB	BB	BB	BB	BB	BB
HI	CC	CC	CC	CC	CC	CC	CC	CC
HK-1	CC	CC	CD	CD	CC	CD	CC	DD
HK-2	CC	CC	CC	CC	CC	DD	DD	DD
PEP-B	BB	BB	BB	BB	BB	BB	BB	n/a
MDH-1	BB	BB	BB	BB	AA	AA	AA	AA
MDH-2	DD	DD	DD	DD	BB	BB	BB	BB
PGM	DD	DD	DD	BCD	DD	BCD	DD	BC
UAE	n/a	BB	n/a	n/a	n/a	n/a	n/a	n/a
LDH	AA	AA	AA	AA	AA	AA	AA	n/a

\* Locus abbreviations are indicated in Table I; n/a = no activity detected.

morphism observed in the present study conflicts with the results of Soliman et al. (1982), who reported variation in 4 enzymes (lactate dehydrogenase, malate dehydrogenase, hexokinase, and phosphohexose isomerase) among clones derived from 32 strains of *T. vaginalis*. These 4 loci were monomorphic in the strains of *T. vaginalis* we studied. Limited variability at the phosphoglucumutase locus was detected by Soliman et al. (1982) and in the present investigation. Clearly, enzyme polymorphism in the *T. vaginalis* isolates we examined was considerably lower than that found by Soliman et al. (1982) or by us for *T. gallinae*. At this time, explanations for such differences remain speculative; however, some possibilities merit discussion. First, the isolates we studied may not be representative of the genetic variability present in *T. vaginalis*. All of the strains and clones we surveyed, except for TvK1, were obtained from patients attending the Johns Hopkins Hospital Gynecologic or Obstetric Clinics in Baltimore, Maryland, U.S.A. A comparison of strains isolated from women residing in distant geographic localities may reveal additional variation. Second, very little is known about what effects *in vitro* cultivation has on the genetic composition of isolates. Culture medium could act as a highly selective environment, or conversely, an unrestrictive one, which supports the growth of mutants that would not normally survive *in vivo*. One possibility is that *T. gallinae*, which usually grows more vigorously than *T. vaginalis in vitro*, may experience less selection pressure or fewer population bottlenecks than *T. vaginalis* upon initial isolation. Thus, variability

of *in vivo* strains of *T. gallinae* may be better preserved upon *in vitro* isolation. It should also be noted that differences at the hexokinase-1 locus between JB6 clone 1 (1970) and clones 7 and 8 may reflect allomorphic variation that was once present in the parent strain from which these clones were derived. Isoenzyme variation among clones of *Trypanosoma cruzi* derived from a single parental stock has been reported (Goldberg and Silva Pereira, 1983).

This investigation also demonstrates that isoenzyme genotypes are not stable attributes of *in vitro*-cultivated *T. gallinae*. From the observations with *T. gallinae* JB6 clones, it appears that *in vitro* mutation influences the extant isoenzyme phenotypes of clones. For example, following an undefined period of *in vitro* cultivation, JB6 cl 1 began expressing a phosphoglucumutase allomorph that was not detected in cultures prepared from the 1970 stabilate of that clone. This allomorph, which was also found in other clones of JB6, probably originated by mutation. Changes in isoenzyme patterns and virulence during axenization of *Entamoeba histolytica* have also been reported (Mirelman et al., 1986).

Changes in the composition of *T. gallinae* populations were observed with SG "clone 1." Isoenzyme study revealed that cultures inoculated with the original SG cl 1 stabilate had a complex BCD phosphoglucumutase phenotype, which implies that it was not a clone, but rather a mixed population. This was substantiated by the subclone SG scl 1 that had a homozygous DD phosphoglucumutase genotype. Only by additional subcloning from SG cl 1 could it be ascertained

TABLE II. *Extended.*

<i>Trichomonas vaginalis</i>			<i>Tritrichomonas foetus</i>			
Balt 41	Balt 42 cl(s) 4, 6, 7, 8, 9, 11 Balt 23, 42, 44, 53, 55, 70 Balt 44 cl(s) 4, 5, 7 JH31-4 JH31-4 cl(s) 1, 3, 5 TvKl	Balt 80	KV-1	COLO cl 1 CB DK-2 UT-1	<i>Pentatricho- monas hominis</i> cl 1	<i>Tetratricho- monas gallinarum</i> cl 1
BB	BB	n/a	n/a	n/a	AA	AA
DD	DD	DD	AA	AA	CC	BB
AA	AA	AA	CC	n/a	CC	CC
BB	BB	BB	DD	DD	AA	CC
EE	EE	EE	AA	AA	BB	DD
EE	EE	EE	AA	AA	BB	FF
BB	BB	AB	BB	n/a	AA	BB
BB	BB	BB	BB	CC	DD	AA
CC	CC	CC	CC	EE	FF	AA
AB	BB	BB	AA	AA	AA	DD
n/a	n/a	n/a	AA	n/a	n/a	n/a
CC	CC	CC	n/a	n/a	BB	CC

how many other genotypically distinct populations are present within this culture. Interestingly, during 12 mo of *in vitro* cultivation, SG cl 1 phosphoglucomutase and hexokinase-1 phenotypes changed. As can be seen from the SGC cl 1 data (Table II), the SG cl 1 mixed population became monomorphic for hexokinase-1 and “heterozygous” for phosphoglucomutase. In this case, no new allomorphs were expressed, and it is unknown whether a subpopulation containing the other allomorphs remains as a hidden component of the SGC cl 1 population.

Within *Trichomonas gallinae* and *Tritrichomonas foetus*, some strains could be definitively diagnosed on the basis of their isoenzyme phenotypes. For example, *T. gallinae* SG, IBERG, and JB6 strains can be differentiated from one another. In addition, the strains and clones of *T. gallinae* that were isolated from virulent stocks differed in their hexokinase-2 genotype from the avirulent SG clones. *Tritrichomonas foetus* KV1 has malate dehydrogenase mobility differences

that differentiate it from all other strains of this species examined. These malate dehydrogenase allomorphs differentiate the highly virulent *T. foetus* KV-1 isolate from moderately virulent and avirulent strains and clones. However, for both *T. gallinae* and *T. foetus*, a limited number of strains with known virulence levels have been examined, and therefore it is premature to conclude that these genetic markers are correlated with virulence. Unfortunately, isoenzymes did not differentiate among *T. vaginalis* strains that varied considerably in their inherent pathogenicity (Honigberg et al., 1984).

The UPGMA phenogram (Fig. 2) shows the relative degree of genetic differentiation among the taxa examined by us, and should not be interpreted as representing phylogenetic relationships. Rogers’ (1972) genetic similarity coefficients among these taxa are very low, which suggests that times of divergence among these taxa are ancient. Phenetic clustering of the genetic data results in associations that are discor-

TABLE III. *Matrices of Rogers’ similarity values (above the diagonal) and Nei’s distance coefficients (below the diagonal) for representative trichomonad taxa.*

Taxon	1	2	3	4	5	6	7	8	9	10
1 <i>Trichomonas gallinae</i> JB6	—	0.944	0.778	0.667	0.348	0.333	0.278	0.333	0.0	0.222
2 <i>Trichomonas gallinae</i> JB6 cl 1 1970	0.029	—	0.722	0.611	0.363	0.348	0.293	0.389	0.015	0.237
3 <i>Trichomonas gallinae</i> IBERG	0.251	0.297	—	0.889	0.237	0.222	0.167	0.444	0.0	0.111
4 <i>Trichomonas gallinae</i> SGcl 1	0.405	0.464	0.118	—	0.237	0.222	0.167	0.444	0.0	0.111
5 <i>Trichomonas vaginalis</i> BALT 41	1.070	1.041	1.475	1.475	—	0.944	0.889	0.126	0.056	0.389
6 <i>Trichomonas vaginalis</i> BALT 42	1.099	1.070	1.504	1.504	0.029	—	0.944	0.111	0.0	0.333
7 <i>Trichomonas vaginalis</i> BALT 80	1.252	1.224	1.763	1.763	0.061	0.029	—	0.056	0.056	0.278
8 <i>Trichomonas gallinarum</i> cl 1	1.099	0.916	0.811	0.811	2.169	2.197	2.862	—	0.111	0.222
9 <i>Pentatrichomonas hominis</i> cl 1					2.862		2.862	2.197	—	0.222
10 <i>Tritrichomonas foetus</i> KV 1	1.504	1.475	2.197	2.197	0.916	1.099	1.252	1.504	1.504	—



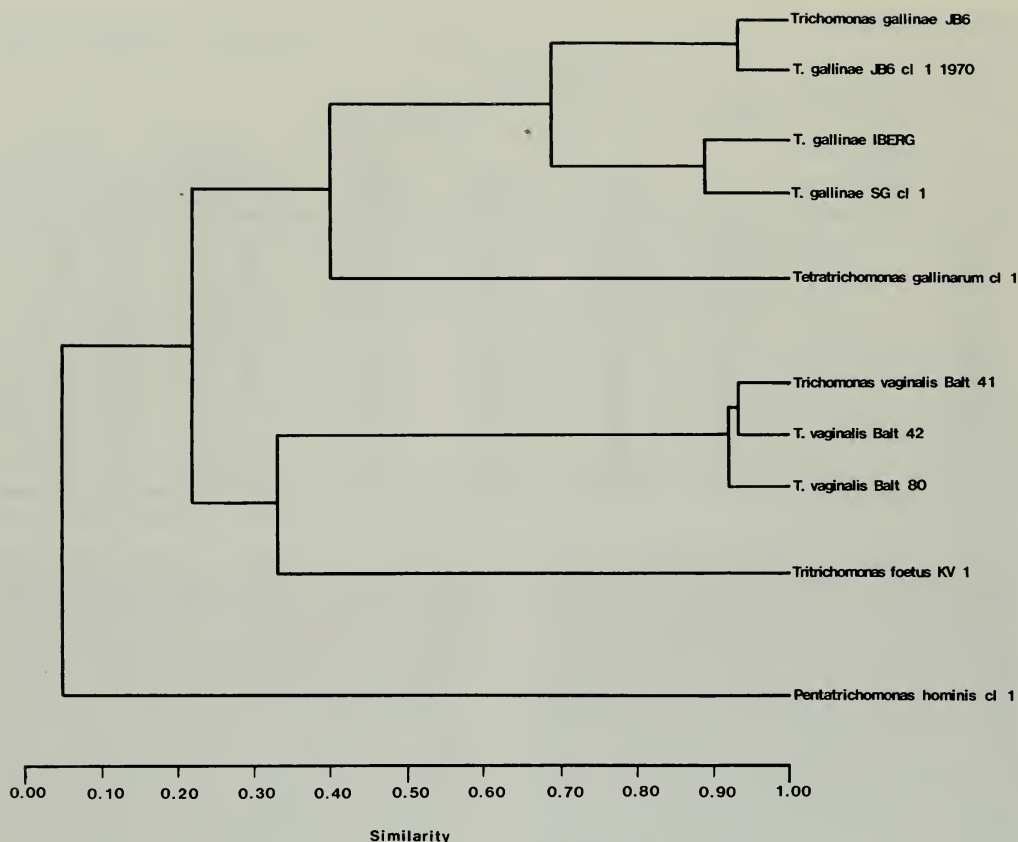


FIGURE 2. Phenogram of genetic differentiation among representative trichomonad taxa. Rogers' similarity metric was clustered by the UPGMA method. The cophenetic correlation coefficient for the phenogram is 0.966.

dant with both alpha-taxonomy and limited immunological data (Honigberg, 1978; Stepkowski and Honigberg, 1981). For example, the congeners *T. gallinae* and *T. vaginalis* do not cluster together in the phenogram, but instead are grouped with *Tetratrichomonas gallinarum* and *Tritrichomonas foetus*, respectively. It is noteworthy that phenetic clustering of the genetic data places the 2 urogenital species from mammals and the 2 species from the digestive tract of birds into 2 distinct groups. However, the low levels of genetic similarity among the species studied indicate that studies of more conservative molecules may be required to clarify the phylogenetic relationships in this group. Finally, the low levels of genetic similarity between *T. gallinae* strains isolated in Colorado and Pennsylvania suggest that there may be significant geographic variation in this species.

Subunit structures of the proteins hexokinase-1 and phosphoglucumutase in *T. gallinae* correspond to those of a phylogenetically diverse array

of animals (Dessauer and Braun, 1982), as do those of other protozoa (Chapman et al., 1984). The expression of 2 allomorphs in equal dosage as observed in heterozygous clones suggests that trichomonads are diploid for these protein loci. However, this does not necessarily imply that they are chromosomally diploid. Studies of a similar nature have led investigators to conclude that *Trypanosoma cruzi* (Tibayrenc et al., 1981; Miles, 1983) and *Trypanosoma brucei* (Gibson et al., 1985; Jenni et al., 1986) are also diploid at many loci coding for enzymatic proteins.

Difficulties in resolving certain loci in some trichomonad taxa (see also Soliman et al., 1982) have many potential causes. No lactate dehydrogenase activity was found in *T. foetus* strains, however a recent report (Steinbuchel and Muller, 1986) indicates that this species does not produce lactate as a metabolic end product. Some enzymatic activities, hexokinase-2 in *T. vaginalis* for example, were lost relatively rapidly following homogenization. Presumably this locus and cer-

tain others were either thermolabile or susceptible to proteinases released on homogenization (Coombs and North, 1983). Activation of trichomonad proteases by dithiothreitol in the homogenizing fluid may also have contributed to the observed reduction of some enzymatic activities.

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## A NEW METHOD FOR DIAGNOSIS OF CHAGAS' DISEASE: DIFFUSION-IN-GEL ENZYME-LINKED IMMUNOSORBENT ASSAY

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**ABSTRACT:** The diffusion-in-gel enzyme-linked immunosorbent assay (DIG-ELISA) for antibodies to *Trypanosoma cruzi* was evaluated using formalinized epimastigotes as antigen. The results obtained by DIG-ELISA were compared with those obtained with the indirect hemagglutination test. The results of the DIG-ELISA showed that the reaction zone diameters obtained with sera from individuals with past or present exposure to *T. cruzi* were significantly greater than those obtained with normal human sera. All the sera from Chagasic individuals had a positive reaction in the test, whereas sera from normal individuals and individuals with toxoplasmosis or cutaneous leishmaniasis had a negative result. A close correlation was observed between the reaction zone diameters and antibody concentration (expressed as  $\log_2$  of the serum dilution). Excellent correlation was observed between results obtained by the 2 serological tests. The data suggest that the DIG-ELISA is a promising serological test for measuring antibodies to *T. cruzi*.

Chagas' disease (American trypanosomiasis), a protozoan infection caused by *Trypanosoma cruzi*, occurs only in the western hemisphere. It is estimated that in Central and South America, 10% of the population is infected with the parasite (Braun and de Titto, 1985). The infection is chronic and frequently the organism cannot be demonstrated; therefore, serology has been an important tool in the diagnosis and the epidemiology of *T. cruzi* infections. A number of serological tests have been used in diagnosing exposure to the organism; however, most of them cannot be performed easily in the field (Kagan, 1980). The ELISA test, which is highly sensitive and specific, has been used to quantitate circulating antibodies to *T. cruzi* in sera (Voller et al., 1975; Anthony et al., 1979; Spencer et al., 1980); however, for field studies, this test is not practical because it requires special instrumentation such as a spectrophotometer for quantitation. Recently, Elwing and Nygren (1979) described the method diffusion-in-gel enzyme-linked immunosorbent assay (DIG-ELISA), which is a modification of the ELISA test. In this method unknown sera are allowed to diffuse through a semisolid medium and form stable immune complexes with the antigen previously adsorbed

onto a plastic surface. The immune complexes are then incubated with a second antibody coupled to an enzyme, and the reaction is detected visually by formation of a colored product after incubation with the substrate for the enzyme.

The present study was designed to evaluate the utility of the DIG-ELISA test for the diagnosis of *T. cruzi* infections in humans. Results using this assay were compared with those obtained by the indirect hemagglutination (IHA) test, which was considered as the reference test.

### MATERIALS AND METHODS

#### Parasite

A strain of *T. cruzi* isolated from a human case of Chagas' disease in Cocula, Jalisco, Mexico, was used as antigen for the DIG-ELISA test. The strain has been maintained in the laboratory since its isolation in 1976 and has been designated the Cocula strain.

#### Sera

Serum samples from 31 patients that were shown to be infected with *T. cruzi* by xenodiagnosis or by repeated positive serological tests (IHA) were tested. Normal serum controls consisted of 32 specimens collected from healthy individuals who had no known previous exposure to *T. cruzi* and were negative when tested by xenodiagnosis. Sera were also collected from 5 patients with toxoplasmosis and 4 patients with cutaneous leishmaniasis. The patients, from whom the sera were collected, resided in various regions of Mexico. Known positive and negative human sera were used as controls in the serological tests and to titrate the antigen and the conjugate for use in the DIG-ELISA test. All sera were stored at  $-20^\circ\text{C}$  in small aliquots.

#### DIG-ELISA

For the DIG-ELISA, epimastigotes were grown in 250-ml Erlenmeyer flasks containing a diphasic medium. The solid phase (50 ml) consisted of BHI medium supplemented with 2% agar and 1% dextrose.

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The liquid phase (50 ml) consisted of 0.95% sterile saline. Each flask was inoculated with approximately  $1.5 \times 10^7$  *T. cruzi* epimastigotes cultured in the same medium as above but the solid phase was supplemented with 10% fresh defibrinated rabbit blood. The flasks were incubated at 28 C until peak growth was reached, usually in 10–12 days. To harvest the parasites, the liquid phase from the flasks was pooled, filtered through a funnel loosely packed with glass wool to remove any particles of agar, and washed 3 times with 0.01 M phosphate-buffered saline (PBS), pH 7.2, by centrifugation at 850 g for 15 min at 4 C. The parasites were then fixed in 1.5% formalin-PBS (pH 7.6) for 1 hr at room temperature (RT) and washed 3 more times with 0.01 M PBS, pH 7.6, by centrifugation at 850 g for 15 min at 4 C.

Polystyrene petri dishes (100 mm diameter) (Laboratorios Technicare, Mexico, D.F., Mexico), previously washed with 15 ml of 70% ethanol for 2 min and air dried, were used as the solid support for the antigen. To adhere the formalin-fixed *T. cruzi* epimastigotes to the dishes, the parasite number was adjusted to  $1 \times 10^8$  cells in 15 ml of carbonate-bicarbonate buffer (15 mM  $\text{Na}_2\text{CO}_3$ , 35 mM  $\text{NaHCO}_3$ ), pH 9.6, and this suspension was added to the ethanol-treated plates and left overnight at 4 C. The antigen suspension was then poured out and the plates were gently rinsed twice with 15 ml of 0.15 M NaCl. While still wet, 15 ml of melted (and cooled to 40 C) 1% noble agar in 0.15 M NaCl containing 1% normal rabbit serum was added to each dish. Twenty circular holes (3 mm in diameter), evenly arranged in 4 rows, were punched in the gel of each dish; 16 of them were used for the unknown sera, and 4 wells were used for known positive and negative human control sera. Fifteen microliters of serum diluted appropriately with PBS were added to the wells of each dish and incubated at RT for 24 hr in a moist atmosphere; then the gel was removed from the plates and the dishes were washed twice with PBS (0.13 M NaCl, 1 mM  $\text{KH}_2\text{PO}_4$ , 8 mM  $\text{Na}_2\text{HPO}_4$ , and 2 mM KCl), pH 7.4, containing 0.05% Tween 20 (PBS-T). After that, the dishes were incubated for 2 hr at RT with 10 ml of sheep anti-human IgG conjugated with horseradish peroxidase (Cappel Laboratories, Cochranville, Pennsylvania, U.S.A.) diluted 1:2,000 in PBS-T and then washed twice with PBS-T. Agarose (0.75 g/100 ml) was melted in citrate-phosphate buffer (0.1 M citric acid, 0.2 M sodium phosphate), pH 5.0, and cooled to 45 C. The substrate mixture (*o*-phenylenediamine, 4 mg/ml, and 0.15% hydrogen peroxide) was then added to the agarose solution and 12 ml were poured into each of the dishes. After 20 min of incubation at RT in the dark, the colored circular areas, consisting of orange-yellow by-products of the enzymatic reaction, were measured with a transparent ruler. All sera were tested in duplicate and the average reaction zone diameter was used.

To determine the optimal antigen ( $1 \times 10^8$  cells/plate) and conjugate (1:2,000) dilutions, parasite suspensions of  $1 \times 10^6$ ,  $1 \times 10^7$ ,  $1 \times 10^8$ , and  $1 \times 10^9$  cells/15 ml of carbonate-bicarbonate buffer, and conjugate dilutions of 1:1,000, 1:1,500, 1:2,000, and 1:3,000 prepared in PBS-T were evaluated by DIG-ELISA using positive and negative control sera. The dilutions that gave the maximum difference between the positive and negative sera were subsequently used.

To determine the relationship between the diameters of the reaction zones and the antibody titers, 2-fold dilutions of 24 serum samples (12 positive and 12 negative sera) were prepared and assayed by DIG-ELISA. In order to test the stability of our antigen, the dose-response experiments were repeated using 1-mo-old presensitized plates instead of fresh sensitized plates.

## IHA

For the IHA test, the *T. cruzi* antigen consisted of tanned human type O erythrocytes sensitized with a *T. cruzi* antigen preparation. This antigen was kindly donated by Dr. Elsa Segura from the Instituto Nacional de Diagnostico e Investigacion de la Enfermedad de Chagas, "Dr. Mario Fatala Chaben" (Buenos Aires, Argentina). The test was performed as described in Actualizacion sobre Enfermedad de Chagas, Leishmaniasis y Paludismo (1985) (manual published by the aforementioned institute). Briefly, doubling dilutions of inactivated sera were prepared in 0.01 M PBS, pH 7.2, and 25  $\mu\text{l}$  of the dilutions were added to the wells of microtitration U plates. Then 25  $\mu\text{l}$  of the sensitized erythrocyte suspension were added to each well, and the plates were incubated 3 hr at RT. A reaction was considered positive when a carpet of cells covered the bottom of the well. In negative tests, the cells settled to form a compact button or ring at the well center. A titer equal to or greater than 16 was considered positive.

## RESULTS

The DIG-ELISA reactions were visualized by the formation of orange-yellow circular areas around the location of the former serum application wells. These circular areas appeared approximately within 1 min after adding the substrate-containing gel. The diameters of the zones increased with increasing incubation time during the first 8–10 min of incubation. After that period of time no apparent increase in the diameters of the reaction zones was observed; however, an increase in the intensity of the color occurred.

The results obtained by DIG-ELISA for detection of antibodies to *T. cruzi* in human sera are summarized in Table I. The reaction zone diameters of the patient sera ranged from 6 mm to 16 mm with an arithmetic mean of 8.67 mm, whereas the reaction zone diameters for the negative control sera ranged between 4 mm and 5 mm with a mean of 4.28 mm. Statistical analysis by the Student's *t*-test revealed a highly significant difference between sera from *T. cruzi*-infected patients and normal human sera ( $P < 0.001$ ). To determine the specificity of this assay, sera from 5 patients diagnosed with toxoplasmosis and 4 patients diagnosed with cutaneous leishmaniasis were tested. The results indicated that the DIG-ELISA is specific for detecting an-

TABLE I. Reaction zone diameters of human sera (obtained from various sources) that were assayed for antibodies to *T. cruzi* with the DIG-ELISA.

Sera*	No.	Reaction zone diameter (mm)		P†
		Range	$\bar{x} \pm SD$	
Patients infected with <i>T. cruzi</i>	31	6–16	$8.67 \pm 2.24$	<0.001
Patients with toxoplasmosis	5	4–5	$4.4 \pm 0.55$	
Patients with cutaneous leishmaniasis	4	4–5	$4.25 \pm 0.50$	
Normal controls	32	4–5	$4.28 \pm 0.45$	

\* Diluted 1:2 in PBS, pH 7.2.

† Probability based on the Student's *t*-test (patients vs. normal controls).

tibody to *T. cruzi*, as these sera gave results comparable to the normal control sera.

Comparison of DIG-ELISA results with the results obtained by the IHA test is shown in Table II. Agreement between these tests was 100%. For the DIG-ELISA, a reaction was considered positive when the reaction zone diameter was  $\geq 6$  mm. This minimum zone diameter (6 mm) of the positive tests was larger than the mean value plus 3 standard deviations of the reaction zone diameter of the normal controls. This minimum zone diameter was also larger than the maximum zone diameter obtained from any of the uninfected control sera. For the IHA test, a titer of  $\geq 16$  was considered positive. The titers of the positive sera ranged from 16 to 2,048, whereas the titers of the negative sera ranged from 2 to 8.

Figure 1 shows the results of the experiments performed to determine the relationship between the diameters of the reaction zone and the antibody concentration in the serum samples. A correlation coefficient of 0.99 was obtained, indicating a close relationship between an increase in the diameter of the reaction zone and an increase in the antibody concentration (expressed as  $\log_2$  of the serum dilution). When the above experiments were repeated using 1-mo-old pre-sensitized plates instead of fresh sensitized plates, a slight decrease in the diameters of the reaction zone of the sera was observed for both positive and normal sera (Fig. 1); however, the difference

between positive and normal control sera was still statistically significant ( $P < 0.001$ ).

## DISCUSSION

A good diagnostic test for Chagas' disease is a top priority in rural health centers throughout Central and South America, including Mexico. This test must be highly specific and sensitive, inexpensive, easy to perform, and capable of

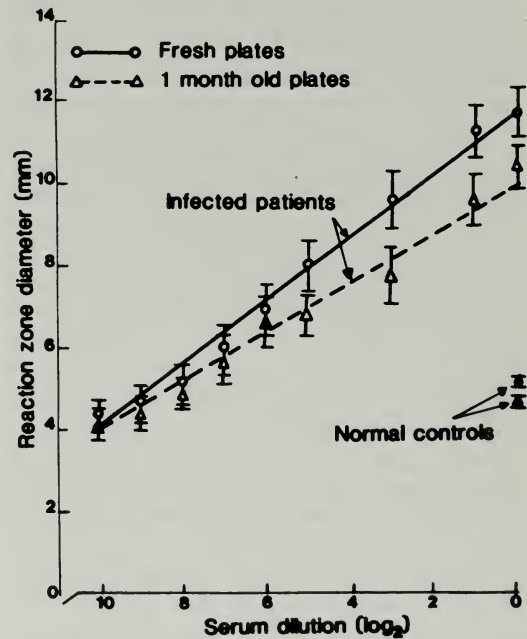


FIGURE 1. Correlation between diameters of the antigen-antibody reaction zones and antibody concentration using the DIG-ELISA. Doubling dilutions of the sera were incubated with formalinized epimastigotes of *T. cruzi* previously adsorbed onto a petri dish. The immune complexes were then detected by the addition of horseradish peroxidase-conjugated sheep anti-human IgG and the enzyme substrate as described in the Materials and Methods section. The experiments were performed using fresh sensitized plates (○—○) or 1-mo-old sensitized plates (△—△). Each bar represents the mean  $\pm$  SE of 12 serum samples.

TABLE II. Comparison of DIG-ELISA and IHA in detection of antibodies to *T. cruzi* in human sera.

	DIG-ELISA*	IHA†
No. of sera positive for <i>T. cruzi</i> antibodies	31	31
No. of sera negative for <i>T. cruzi</i> antibodies	32	32
Total sera tested	63	63

\* Sera with zone diameters  $\geq 6$  mm were considered positive.† Sera with titers  $\geq 16$  were considered positive.



handling a large number of specimens under the most adverse environmental conditions.

The results presented here indicate that the DIG-ELISA test may be useful for a rapid and accurate demonstration of antibodies of *T. cruzi*. The sensitivity of the test in terms of its ability to detect infected individuals appears to be excellent. All patients with a positive xenodiagnosis and/or repeated positive serology (by IHA) had a positive result in the DIG-ELISA test, whereas negative results were found in all the sera obtained from healthy controls and in the sera obtained from patients with toxoplasmosis or cutaneous leishmaniasis. The excellent correlation observed between results obtained by the DIG-ELISA and those obtained by the IHA test suggests that the DIG-ELISA might compare with the standard serologic test in the serodiagnosis and seroepidemiology of Chagas' disease. Nevertheless, further evidence is still necessary to support this conclusion.

The interpretation of results in DIG-ELISA is very simple because only the diameter of the reaction zone has to be considered. Using the horseradish peroxidase system, the reaction zones appeared as circular areas with well-demarcated margins. The areas were easily visualized because of the high contrast of the intense orange-yellow color of the reaction zone against the light background. These characteristics allowed measurement of the zone diameters directly on the plates using a transparent ruler. In the conventional ELISA, the enzymatic reaction is usually stopped by adjusting the pH. In DIG-ELISA there is no need to stop the reaction because the diameter of the reaction zone is not significantly influenced by the enzyme-substrate reaction time. However, after 1 hr of incubation a decrease in contrast occurs due to spontaneous conversion of the substrate (Elwing et al., 1980) and perhaps some diffusion of the converted substrate out of the reaction zones. The 20-min incubation period for the substrate used by Elwing et al. (1980) in other antigen-antibody systems, and also used in our study, was satisfactory. Twenty minutes produced sufficient conversion of the substrate to the colored products to accurately read the assay, but was not enough time for the zone limits to deteriorate and possibly influence reading of the test.

We showed that the diameter of the reaction zone is directly proportional to the antibody concentration in the serum tested. Therefore, given a standard reference serum, antibody concentra-

tion can be estimated directly from the diameter of the reaction areas eliminating the need for serum dilutions. This feature represents an advantage of DIG-ELISA over other serological tests used in the diagnosis of *T. cruzi* infections such as complement fixation, IHA, direct agglutination, ELISA, etc. (Kagan, 1980), which require serum titration. Another advantage of DIG-ELISA over the traditional microplate ELISA technique is that the former does not require a spectrophotometer to read the results, making DIG-ELISA a more practical technique for field studies and for laboratories without easy access to special instrumentation.

The antigen used to perform the DIG-ELISA consisted of whole formalin-fixed cultured epimastigotes adsorbed onto a petri dish. The use of formalinized epimastigotes introduces an advantage over previously reported ELISA techniques for the diagnosis of Chagas' disease (Voller et al., 1975; Spencer et al., 1980), because formalinized antigens are easier to prepare and can be stored for prolonged periods of time at 4 C (Araujo and Guptill, 1984). Preparation of lysates of organisms may be time-consuming and potentially dangerous if not properly done. Furthermore, the lysed antigens must be kept lyophilized or frozen at low temperatures to minimize protein degradation by proteases of parasite origin (Castilla-Garcia, 1984). We tested the activity of our antigen after 1 mo of storage at 4 C, and although a decrease in the zone reaction diameters was observed, the difference between the 2 groups of sera (positive and negative) was significant. The observed decrease may be due to a loss of activity during storage of either our antigen or the sera tested. Therefore, further studies are required to accurately determine the shelf life of the antigen.

Although the use of formalinized epimastigotes is an advantage of the DIG-ELISA presented here, further studies to prove specificity of this particular antigen, and consequently of the assay, must be performed. It has been shown by Gam and Neva (1977) that the use of crude antigens may increase the chance of serologic cross-reactivity. This cross-reactivity occurs as a consequence of shared antigens between related or unrelated species and results in false-positive assays. Our assay appears to be specific for the detection of antibodies to *T. cruzi*; however, the number of sera tested from patients with other protozoan infections was limited. The possibility of having an assay that does not cross-react with

antibodies to other parasites, particularly with antibodies to *Leishmania*, will be an important contribution to the immunodiagnosis of Chagas' disease.

Biological differences among strains of *T. cruzi*, inherent or acquired by passage, are well recognized (reviewed by Brener, 1985; Segura and Cazzulo, 1985). Therefore, the possibility of obtaining different serologic results with different strains cannot be excluded. In our study, the strains of *T. cruzi* used to prepare the antigens for DIG-ELISA and IHA were different; however, the correlation between the results of both tests was excellent. These results agree with those obtained by Spencer et al. (1980), who found no difference in complement fixation results with various strains of *T. cruzi* as antigen.

In summary, the DIG-ELISA method for detection of antibodies to *T. cruzi* presented here is simple to perform, sensitive, specific, relatively inexpensive, and the results are quantitative. The interpretation of results is very simple because they depend only on visual readings of the reaction zone diameters; therefore, there is no need for a spectrophotometer to read the DIG-ELISA results. Furthermore, in DIG-ELISA it is possible to obtain the titer of antibodies without making dilutions of the serum. This direct titration simplifies the procedure. In addition, the petri dishes used to immobilize the antigens are easy to handle during coating and washing and a large number of sera can be assayed simultaneously. It may be possible to sensitize many plates and store them for weeks without a significant change in the activity of the antigen. The presensitized plates could also be shipped in an ice box to field laboratories where the test could easily be performed. For these reasons, the DIG-ELISA test may be particularly useful for field surveys; however, further work is needed to determine the utility of the method as a routine test for diagnosis of Chagas' disease. Studies that include standardization of the test, sensitivity, and cross-reactivity with other parasites are currently in progress.

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## ANTIBODY PROFILES BY EITB AND ELISA OF CATTLE AND SHEEP INFECTED WITH *FASCIOLA HEPATICA*

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**ABSTRACT:** In evaluating potential mechanisms of immunity in fascioliasis we compared the time-course analysis of the antibody responses between a resistant (cattle) and a susceptible model (sheep). Sera from sheep and cows experimentally infected with *F. hepatica* were reacted with both somatic (FhWWE) and excretory-secretory (ES) antigens in order to evaluate the antibody repertoires in the 2 different hosts. Analysis of these sera by ELISA showed a significant increase in antibody levels by 2 wk in most infected cattle using both somatic and ES antigens, whereas with most infected sheep antibodies are not clearly detected until week 4. By EITB, both infected sheep and cows recognize major somatic polypeptides in a molecular weight range of 30-38 kDa by 8 wk. Cattle recognized 3 additional major antigens of 56, 64, and 69 kDa as early as 6 wk. Various polypeptides of 20-25 kDa are prominently detected by most sheep and very faintly, if at all, by the cow sera. The sera from both sheep and cows also identify ES polypeptides of 20-28 kDa. The patterns of polypeptides recognized by sheep infected with *S. mansoni* and challenged with *F. hepatica* in EITB are almost identical to those with a simple *F. hepatica* primary infection. No significant differences were detected in the antibody kinetics in ELISA between these 2 groups. Differences and similarities between these models could eventually help determine which antibodies may be predictive of resistance or susceptibility in fascioliasis.

*Fasciola hepatica* has a wide vertebrate host range, but is mainly a parasite of domestic ruminants (cattle, sheep, goats), causing a great deal of economic loss throughout the world. At present, fundamental questions regarding the mechanisms of immunity to infection with *F. hepatica*, i.e., the site of attrition of a challenge infection and antibody or thymus dependency of immunity, still remain to be answered.

Cattle acquire resistance to challenge infection with *F. hepatica* when they are sensitized by primary or drug-abbreviated homologous infections (Ross, 1966; Boray, 1969; Doyle, 1971, 1973). This resistance was confirmed by Doyle (1972), who showed that rejection occurs by 24 wk after a primary infection and that there is a direct relationship between duration of primary infection and resistance to challenge (Doyle, 1973; Doy and Hughes, 1984). Recent studies done by recovering challenge flukes from either the liver or body cavity appear to indicate that resistance mechanisms against challenge flukes are effective at or soon after penetration of the liver (Doy and Hughes, 1984).

The nature of the resistance to a challenge infection in cattle is probably multifactorial, and both physical barriers and specific acquired immune mechanisms have been implicated. In rats, the classic model for acquired resistance in fascioliasis, a number of reports have shown successful transfer of immunity to *F. hepatica* using serum from homologous and heterologous donors (Dargie et al., 1973; Armour and Dargie, 1974; Hayes et al., 1974; Haroun et al., 1981). In one experiment Haroun et al. (1982) reported that absorption of serum from *F. hepatica*-infected cattle with culture fluid containing metabolic products from adult *F. hepatica* removed its ability to protect rats from a homologous challenge.

There is no evidence to indicate that primary sensitization with *F. hepatica* stimulates any resistance to challenge in sheep, and thus, they are considered susceptible hosts (Sinclair, 1975; Rushton, 1977; Knight, 1980; Sandeman and Howell, 1981). Resistance to *F. hepatica* challenge infection is clearly host dependent: cattle, rats, and goats have been found to develop significant levels of resistance, whereas sheep and rabbits do not (Haroun and Hillyer, 1986).

Previous studies of ours have employed immunoprecipitation and the enzyme-linked immunoelectrotransfer blot (EITB) technique to identify somatic and excretory-secretory antigens that are recognized by the sera from experimentally infected rabbits at different intervals of infection (Santiago et al., 1986). The present

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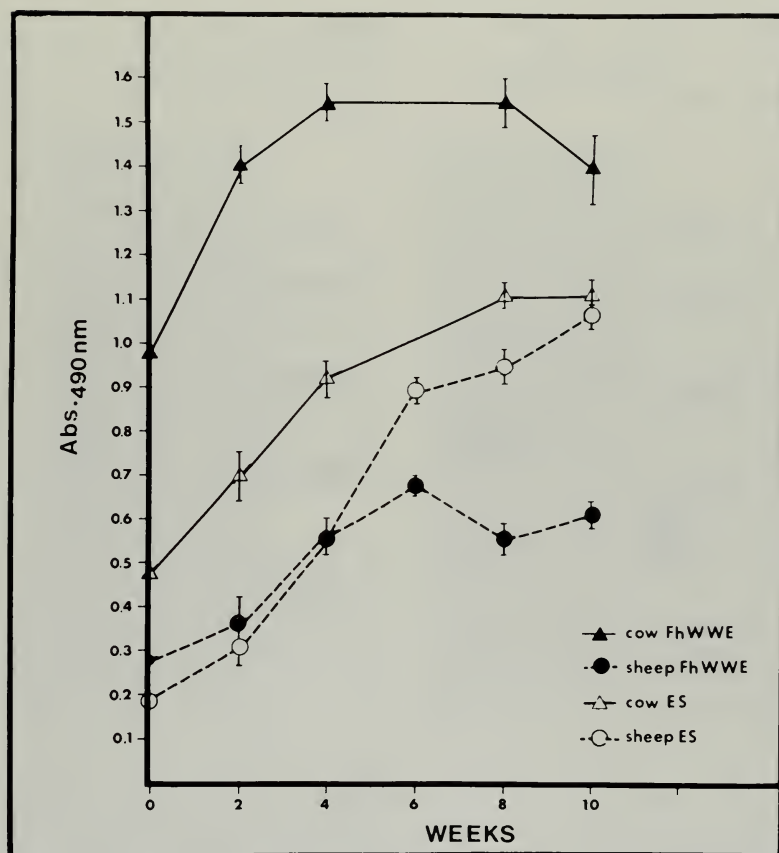


FIGURE 1. ELISA of the sera from cows and sheep infected with *F. hepatica* and bled through 10 wk of infection reacted with both somatic and ES antigens. Profiles represent the average absorbance values obtained with the serum from 5 cows and 5 sheep. Each value shows standard errors obtained.

studies involve an EITB analysis of sera from experimentally infected cattle and sheep in order to study the antibody specificities in the 2 hosts using both somatic and excretory-secretory *F. hepatica* adult antigens. The antibody response in these 2 hosts was also measured by ELISA. Previous work in our laboratory has examined the induction of protective mechanisms by immunizing with heterologous (*Schistosoma mansoni*) infections (reviewed by Hillyer, 1984). Preliminary studies have shown that mice infected with *S. mansoni* or calves infected with *S. bovis* develop partial protection to challenge infection with *F. hepatica* (Christensen et al., 1978, 1980; Sirag et al., 1981; Hillyer, 1984). In the present study we also compare the antibody reactivities of sheep with primary fascioliasis versus sheep that have been first exposed to *S. mansoni* and then infected with *F. hepatica*. Differences and similarities between these 2 models could even-

tually help determine antigens that could be relevant to the development of protective immune mechanisms.

## MATERIALS AND METHODS

### Preparation of antigens

**Excretory-secretory (ES) antigens:** Live, intact adult *F. hepatica* worms were obtained from bovine livers at a local abattoir and washed 3–4 times at room temperature during 1 hr with 0.01 M phosphate-buffered saline (PBS), pH 7.2, to remove all traces of blood and bile. The worms were then incubated (20 worms/100 ml) in PBS at 37 C for 3 hr. The supernatant was then centrifuged at 4 C, 20,000 rpm for 1 hr to remove particulate material.

**Somatic antigens (FhWWE):** Adult *F. hepatica* worms were obtained from bovine livers, washed, and homogenized in a Waring blender. The homogenate was then lyophilized and stored at -20 C. Before use, the antigens were again homogenized in a Ten-Broek tissue grinder in an ice bath for 15 min. The homogenized antigens were centrifuged for 15 min in a Beckman microfuge, and the supernatants obtained were



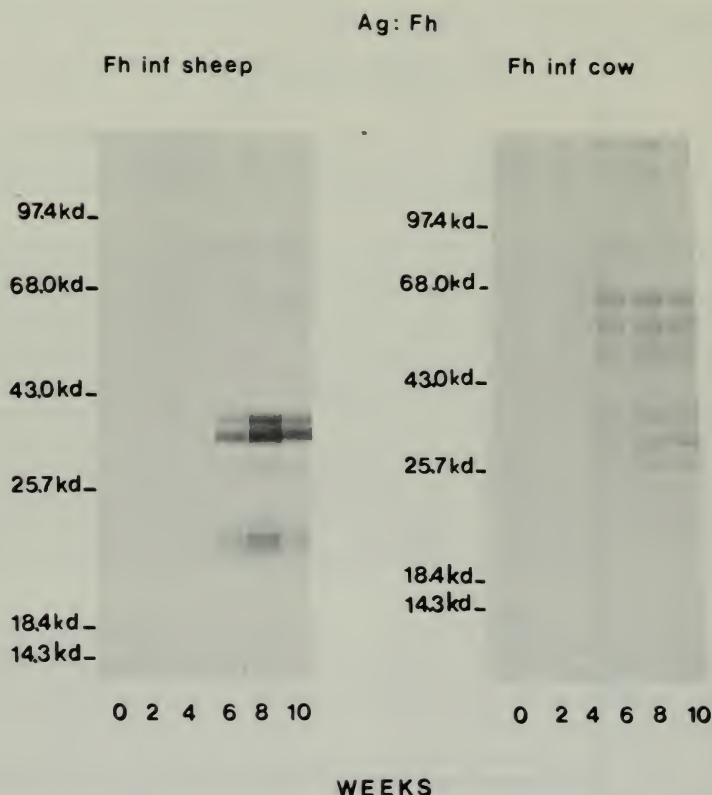


FIGURE 2. EITB of pooled sera from cows and sheep infected with *F. hepatica* reacted with the homologous somatic antigen. Numbers 0–10 represent weeks postinfection.

used in the different assays after determination of their protein concentration by BioRad.

All antigen preparations were stored at  $-20^{\circ}\text{C}$  until used.

#### Preparation of sera

**Cows:** Five Holstein calves, 6 wk old, were infected orally with 700 metacercariae and bled by the jugular vein every 2 wk through 10 wk of infection. The sera were stored at  $-4^{\circ}\text{C}$  until used.

**Sheep—primary infection:** Five sheep, 5 wk old, were infected orally with 400 metacercariae. Five additional sheep infected with 5,000 *S. mansoni* cercariae for 10 wk were infected with 400 metacercariae of *F. hepatica* 10 wk later and bled serially through an additional 10 wk after infection with *F. hepatica* (20 wk after *S. mansoni* infection for the second group). In all cases, sera were obtained by bleeding the animals from the jugular vein. Sera were stored at  $-70^{\circ}\text{C}$  until used. All animals were necropsied 10 wk after being infected with *F. hepatica*.

#### Enzyme-linked immunosorbent assay (ELISA)

The ELISA was performed essentially as described previously (Santiago et al., 1986). Optimum antigen concentration for ES antigens, determined by block titration, was  $2\text{ }\mu\text{g/ml}$ . Antigen was diluted in carbonate buffer, pH 9.6, and incubated for 1 hr at  $37^{\circ}\text{C}$  and

overnight at  $4^{\circ}\text{C}$ . Sera were used at 1:200 in 5% dried milk (blotto), and horseradish peroxidase-conjugated goat anti-rabbit IgG (Miles Laboratories, Elkhart, Indiana) was diluted in 1:100, 0.3% PBS/Tween 20 (which was also used for all washings). Anti-bovine or anti-sheep IgG (KPL) were used at a 1:1,000 dilution in PBS/Tween 20. Incubations were done at  $37^{\circ}\text{C}$  for 1 hr. O-phenylene-diamine was used as substrate and was incubated for 30 min at room temperature ( $25^{\circ}\text{C}$ ) in the dark. An 8 N  $\text{H}_2\text{SO}_4$  solution was added to terminate the reaction ( $25\text{ }\mu\text{l/well}$ ) before the plates were read spectrophotometrically in a Dynatech MR 560 ELISA reader. Final volume of all reactants was  $100\text{ }\mu\text{l}$ .

#### Enzyme-linked immunotransfer blot (EITB)

The EITB was done as described by Tsang et al. (1983). Polyacrylamide gradient gels (5–20%) were electrophoresed in a discontinuous buffer system J 4179 (Jovin et al., 1971) as modified by Neville et al. (in Tsang et al., 1983). Antigen samples were prepared at  $1\text{ }\mu\text{g}/\mu\text{l}$  in a sample buffer of 0.01 M Tris, 2.5% SDS and were heated at  $65^{\circ}\text{C}$  for 15 min.

*Fasciola hepatica* ES antigens ( $150\text{ }\mu\text{g}$ ) were run in a single trough 5–20% SDS-PAGE gradient gel at constant current ( $25\text{ mA/75-mm}$  gel). Transfer of proteins from gels to nitrocellulose sheets was performed at 100 V for 2 hr. The nitrocellulose was washed for 30 min

in blotto, cut into strips, and incubated in 1:200 test serum diluted in blotto followed by incubation in a 1:1,500 dilution of a horseradish peroxidase-conjugated goat anti-bovine or sheep IgG in 0.3% PBS/Tween. Strips were developed by the addition of 3,3'-diaminobenzidine tetrahydrochloride (Sigma).

## RESULTS

In ELISA, using both crude and ES antigen preparations, an increase in antibody levels ( $2\times$  normal) was observed by 2 wk of infection in cows with primary fascioliasis; peak absorbance values were obtained at 8–10 wk postinfection (Fig. 1). Higher means for peak absorbance values were obtained with the ES antigen versus those obtained with somatic antigens. In addition, higher backgrounds were obtained with the crude somatic antigens (Fig. 1).

In sheep, increases in antibody levels were not evident until after 4 wk postinfection (Fig. 1). Although absorbance levels increased to 5 times the normal serum values with ES antigens by 8 wk, the increase in mean absorbance values with the somatic antigen was less dramatic ( $3.5\times$  normal).

Figure 2 shows the somatic antigens recognized by the sera from sheep and cows throughout a 10-wk *F. hepatica* infection reacted in EITB. Both sheep and cow sera recognize major polypeptides of 30–38 kDa by 4–6 wk of infection. In the case of sheep, 2 very prominent antigens of 33 and 38 kDa are consistently detected by all the sheep tested. Four to 5 polypeptides of 20–25 kDa are prominently detected by all 5 sheep tested and very faintly, if at all, by the cow sera (Fig. 2). In addition, 3 polypeptide clusters, which appear as diffuse bands with molecular weights of approximately 56, 64, and 69 kDa, appear to be recognized by the cow sera since early fascioliasis, but are not detected in EITB by the sheep sera tested.

Two of the 5 sera from infected cows and 4 of the 10 sheep sera tested also recognize a group of antigens of 9–14 kDa that resolve as up to 7 distinct polypeptides (Fig. 3). These antigens are found in both somatic and ES antigen preparations and are clearly recognized by the pooled cow sera taken at 10 wk of infection.

Both the sera from sheep and cows with primary fascioliasis detect various major ES antigenic polypeptides ranging from 23 to 30 kDa (Fig. 4). All of the previously mentioned antigens were recognized throughout the duration of the infection.

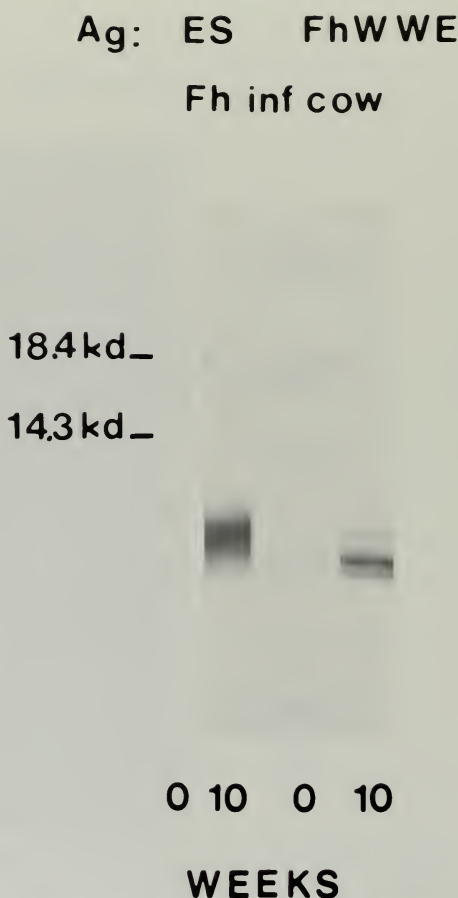


FIGURE 3. EITB of pooled sera from *F. hepatica*-infected cows 10 wk postinfection reacted with the homologous somatic antigen. Only the low molecular weight antigens recognized are shown.

### Primary Fh versus Sm-Fh infections (sheep)

Both the sera from sheep with primary fascioliasis (Fig. 5, open circles) versus those previously infected with *S. mansoni* and then challenged with *F. hepatica* (closed squares) follow similar patterns of reactivity to somatic antigens. Peak absorbance values were obtained at 6–10 wk postinfection. Similar responses were observed in the challenged versus primary infection sheep at the same postinfection period.

Analogous results were obtained in ELISA with the ES antigens. Again, similar patterns of reactivity were observed. In both groups, the increase in antibody levels was more dramatic with the ES than with the somatic antigens. These sera were also analyzed by EITB. When the sera from sheep infected for up to 10 wk with *S. mansoni* are reacted with somatic *F. hepatica* antigens, no



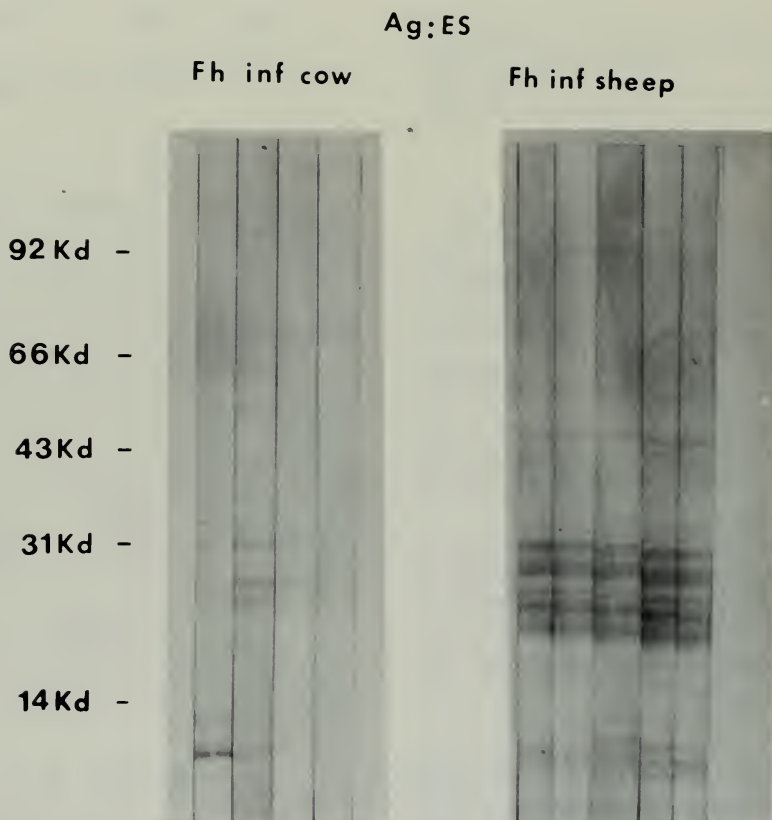


FIGURE 4. EITB of sera from 4 cows and 5 sheep infected with *F. hepatica* and bled at 10 wk postinfection reacted with *F. hepatica* ES antigens.

bands are detected. When these sheep with 10-wk-old *S. mansoni* infection are then challenged with *F. hepatica*, bled through an additional 10 wk and reacted with this antigen preparation, no differences are observed between these and the sera from sheep with a simple primary *F. hepatica* infection. Sera from both the sheep with the challenge *Fasciola* infection and those with primary fascioliasis detect the same major polypeptides in the somatic antigen preparations (data not shown).

The reactivity of sera from sheep with primary fascioliasis versus the *Fasciola*-challenged sheep when reacted with ES antigens was also examined (Fig. 6). The pattern of polypeptides recognized is almost identical between these 2 groups at approximately the same postinfection periods.

#### DISCUSSION

Cattle and sheep have been proposed as resistant versus susceptible hosts for *F. hepatica*. Although cattle are infected, they have been shown

to acquire resistance to challenge infections when sensitized by primary or drug-abbreviated infections (reviewed in Haroun and Hillyer, 1986). In the case of sheep, there is no evidence to indicate that primary sensitization with *F. hepatica* worms by infection or antigen by immunization stimulates any resistance to challenge in terms of reduction in the number of worms recovered from challenge infections. In our laboratory and others (Christensen et al., 1978, 1980; Sirag et al., 1981; Hillyer, 1984), partial protection to challenge infection with *F. hepatica* has been achieved by infecting mice with *S. mansoni* previous to challenge.

The present study compares the antibody response and its specificities in *F. hepatica*-infected cows and sheep, and in sheep with primary versus challenge (previous *S. mansoni*) infections using both somatic and ES antigens in the ELISA and the EITB. The antibody profiles obtained with the sera from infected sheep reacted with somatic and ES antigens is similar to that ob-

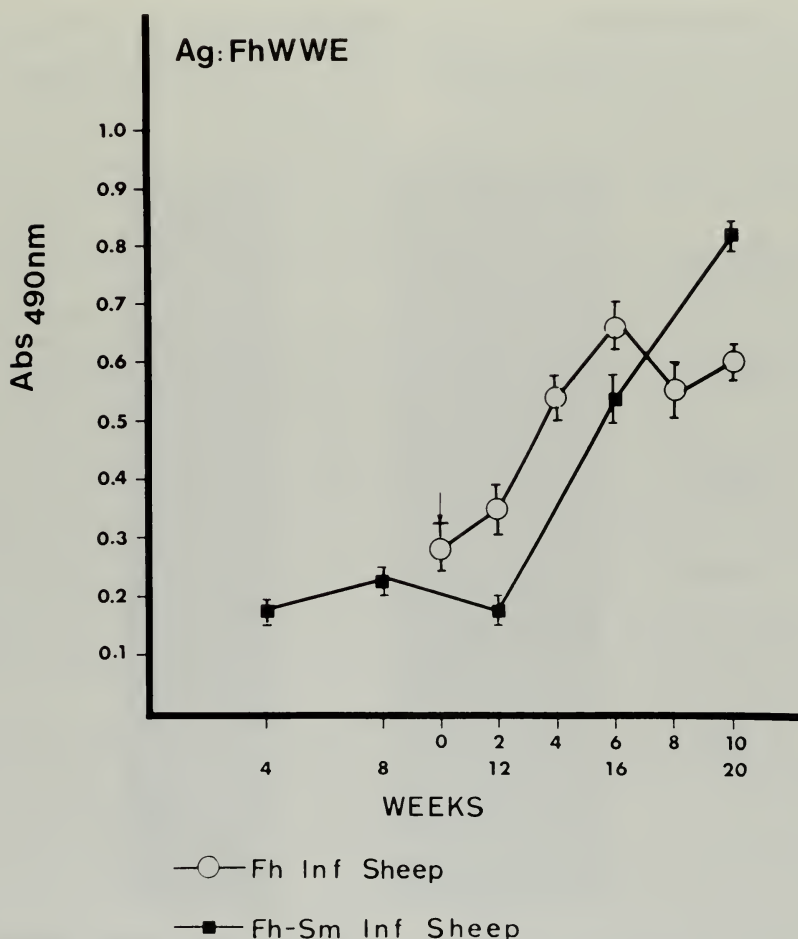


FIGURE 5. ELISA antibody profiles of sera from sheep with primary fascioliasis (Fh inf) versus those previously infected with *S. mansoni* and then challenged with *F. hepatica* (Fh-Sm inf). The sera were reacted with *F. hepatica* somatic antigen (FhWWE).

tained by Movsesijan and Jovanovic (1975) using sera from *F. hepatica*-infected sheep and reacted with whole, fixed adult worms in an immunofluorescence assay. It also concurs with the profile observed by Zimmerman et al. (1982) when measuring total immunoglobulin to somatic extracts during the course of infection. Lehner and Sewell (1980), however, found no differences between somatic and ES antigens when reacted in ELISA with the serum from sheep infected with *F. hepatica* for 8 wk. Differences in the way the ELISA assays are performed in both studies in terms of antigen preparation and concentration may account for these differences.

In cows, the antibody patterns obtained in the present study are analogous to those obtained by Hughes et al. (1981) using sera from *F. hepatica*-infected calves and measuring antibody titers to

gut *F. hepatica* antigens by immunofluorescence. The increase in absorbance values by 2 wk obtained in the present study with the ES antigens may reflect the presence of antibodies to early ES products (i.e., antigens in T1 type granules). The peak at 8–10 wk suggests that antibodies to later secretory products (i.e., from T2 type granules) may also be present.

In general, cows appeared to have sharper increases in antibody reactivity by 2 wk of infection; these increases were clearly observed with both somatic and ES antigens. We have, however, to consider the fact that we are comparing sets of data obtained with 2 different conjugates that probably have different enzyme labeling ratios and that may account for the differences observed. In sheep, an increase in antibody reactivity was not observed until 4 wk postinfection.



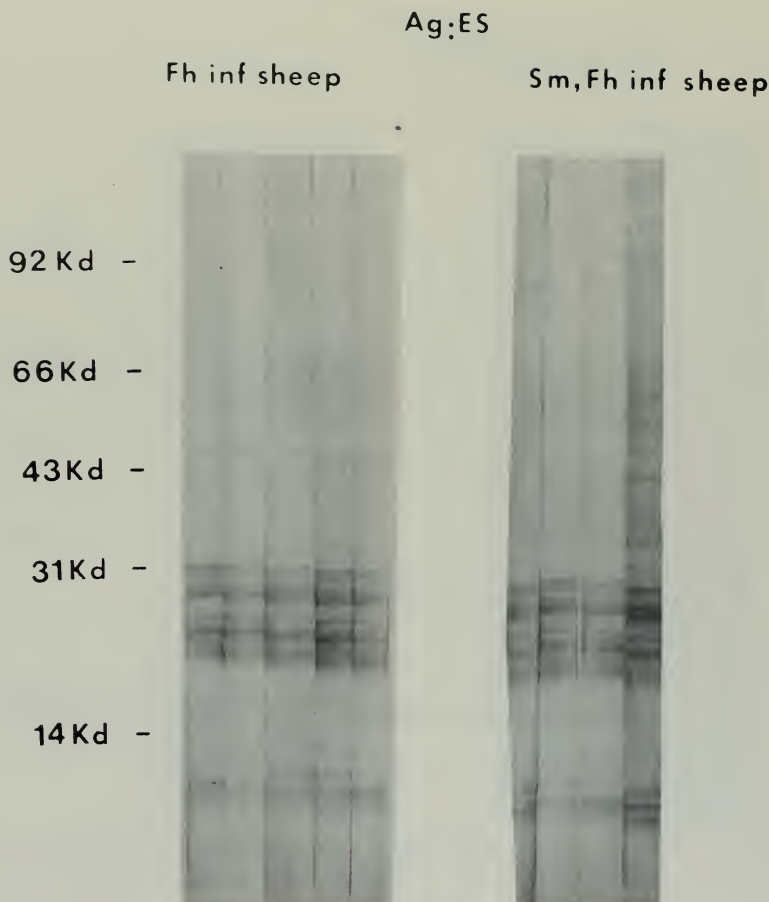


FIGURE 6. EITB of sera from 5 sheep with primary fascioliasis (left panel) versus 4 previously infected with *S. mansoni* and then challenged with *F. hepatica* (right panel). Sera were taken 10 wk after *F. hepatica* infection. The challenged group had been exposed to *S. mansoni* cercariae 10 wk before infection with *F. hepatica*.

It is possible that this lack of reactivity early in infection may in part contribute to the successful migration of juveniles in sheep. Data obtained by Doy and Hughes (1984) recovering immature flukes from either the liver or body cavity after a challenge infection suggest that, in cattle, resistance mechanisms are effective against challenge flukes at or soon after penetration of the liver.

Previous studies in mice and cattle have shown partial protection to *F. hepatica* induced by a previous schistosome infection. In the present study we have also compared the antibody specificities and kinetics of sheep with primary fascioliasis versus sheep that have been exposed to *S. mansoni* previous to infection with *F. hepatica*.

The fact that no anamnestic response was de-

tected in the *S. mansoni*-infected *F. hepatica*-challenged sheep could be due to the fact that sheep do not appear to be good hosts to the schistosome infection. The *S. mansoni*-infected animals had very low schistosome egg counts and were apparently self-cured by 14–16 wk post *S. mansoni* infection (Haroun and Hillyer, 1986). Because recent studies from our laboratory have found *S. mansoni*–*F. hepatica* cross-reactivity associated with egg production in *S. mansoni*-infected animals, it may be that these animals were not exposed to appropriate Sm-Fh cross-reactive epitopes during this abbreviated period of infection with *S. mansoni*.

In both sheep and cattle, the antigens prominently recognized in the somatic preparations are not the same as those prominently recognized in the ES product preparation. In the somatic an-

tigens, both sheep and cows recognize prominent antigens from 30 to 38 kDa, whereas the most prominent antigens recognized in the ES antigen preparation was a group of polypeptides of approximately 23–28 kDa. These 23–38-kDa polypeptides have been found to be recognized by rabbits with fascioliasis, especially in the acute phase of the infection, and constitute the major proteins found in this antigenic preparation (Santiago et al., 1986).

The 3 diffuse bands (56, 64, and 69 kDa) recognized by bovine sera but not detected by sheep sera could be related to the resistance to infection observed in cattle. The group of 9–12-kDa antigens is very distinctly identified by 2 of the 5 cows tested and 4 of the 10 sheep. The development of antibodies to these low molecular weight antigens may depend on the genetic constitution of the host: they are either prominently recognized or not at all. This is of special interest in lieu of the fact that a 12-kDa *F. hepatica* antigen has been shown to induce partial protection to *S. mansoni* challenge in mice (Hillyer et al., 1987).

Antibodies have been involved in resistance mechanisms to *Fasciola* as well as to other trematodes (Haroun et al., 1981). Passive resistance appears to be influenced by the species of the donor and recipient. Baalawy (1975) reported a low level of resistance by transfer of immune homologous serum to rabbits, but immune serum from goats did confer high levels of protection to rabbits. Haroun et al. (1981) also found that rats, rabbits, or cattle can develop significant resistance to challenge with *F. hepatica* and that although immune sera from rats or cattle protect rabbits, immune serum from the homologous host was ineffective in this animal model. Although the differences of antibody specificities between sheep and cattle shown in this study are not dramatic, they could be important, not only for the identification of potential protective antigens, but to identify antigens that may stimulate suppression (i.e., in sheep). These could, in turn, aid in the long-term goal of developing an effective vaccine for this disease.

#### ACKNOWLEDGMENTS

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## ADOPTIVE CELL TRANSFER STUDIES TO EXAMINE THE ROLE OF T LYMPHOCYTES IN IMMUNITY TO *TRYPANOSOMA MUSCULI*

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**ABSTRACT:** Previous studies in this laboratory utilizing monoclonal antibody-induced immunosuppression have demonstrated that the T-helper lymphocyte is primarily responsible for the T lymphocyte dependency of *Trypanosoma musculi* elimination from the bloodstream of mice, and that T-cytotoxic lymphocytes play a minimal role in this response. In the current study, these findings were extended by examining the effects of adoptive cell transfers on the course of infection with *T. musculi* using immune splenocytes enriched for T lymphocyte subpopulations. These studies demonstrated that adoptive transfer of immune splenic T lymphocytes resulted in a specific, dose-related enhancement of kinetics of trypanosome elimination. This effect was found to be due to the presence of L3T4<sup>+</sup> T-helper cells in the immune splenocyte population. Adoptive transfer of Lyt-2<sup>+</sup> T-cytotoxic cells or lymphokine-activated killer (LAK) cells was ineffective in altering the course of infection. In addition, it was found that immune B lymphocytes were equally capable of adoptively transferring immunity to *T. musculi*, suggesting that the primary role of the T-helper lymphocyte is to provide help in the induction of parasite-specific antibodies.

*Trypanosoma musculi* is a hemoflagellate uniquely parasitic for mice (Viens, 1985). The kinetics of intraperitoneal (i.p.) infection with this organism in female B6C3F<sub>1</sub> mice follows a characteristic course with a prepatent period (3-4 days), a logarithmic growth phase (parasites in the blood), a plateau phase (nondividing parasites in the bloodstream), and finally a rapid elimination of trypanosomes from the circulation. Mice eliminating trypanosomes from the circulation are immune to reinfection with *T. musculi*, although some parasites remain sequestered in certain capillaries of the kidney for the life of the animal.

Although infection with *T. musculi* follows a highly defined course in normal mice as described above, the host/parasite system is sensitive to alterations in immunocompetence. Studies with naturally athymic (nude) mice (Brooks and Reed, 1977; Rank et al., 1977) and mice experimentally rendered T lymphocyte deficient (Viens et al., 1975; Pouliot et al., 1977; Targett et al., 1981) have demonstrated that clearance of *T. musculi* from the bloodstream depends upon the presence of functional T lymphocytes. However, these early studies defined neither the precise role of the T lymphocyte, nor the identity of the subpopulation(s) involved in

parasite elimination. Although most of these studies involved the use of adoptive cell transfers to characterize the role of various cell types in this elimination, all of the techniques suffered from a lack of purity in the cell population transferred. The advent of monoclonal antibody reagents (Köhler and Milstein, 1975) has provided researchers with the ability to isolate homogeneous lymphocyte subpopulations, facilitating the adoptive transfer of highly specific phenotypes.

In a previous report describing *in vivo* monoclonal antibody-induced depletion of T lymphocyte subsets (House and Dean, 1988), evidence was presented that the T lymphocyte dependency of *T. musculi* elimination from the circulation is due to the action of T-helper lymphocytes rather than by direct cytotoxic activity of Lyt-2<sup>+</sup> T-cytotoxic cells. The purpose of the present study was to confirm, through adoptive cell transfer experiments, the lack of effect by T-cytotoxic cells, as well as to define further the role of the T-helper phenotype in elimination of *T. musculi* from the bloodstream.

### MATERIALS AND METHODS

#### Mice

Specific pathogen-free female B6C3F<sub>1</sub> (C57Bl/6 × C3H F<sub>1</sub>) mice, 4-8 wk old and weighing approximately 20 g, were obtained from Charles River Breeding Laboratories, Raleigh, North Carolina. Animals were housed on hardwood shavings bedding in filter-top plastic cages and given free access to rodent chow (NIH-07, Ziegler Bros., Gardner, Pennsylvania) and purified tap water. Randomly selected mice were necropsied on arrival (standard screening necropsy; Microbiological Associates, Bethesda, Maryland), and sentinel animals were bled at weekly intervals for murine viral serology.

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All mice were found to be free of known rodent pathogens throughout the study.

### Parasites

The Partinico II strain of *Trypanosoma musculi* was obtained from the American Type Culture Collection (ATCC, Rockville, Maryland). It was shipped in diphasic blood agar and was tested for specific rodent pathogens by a standard screening assay (Microbiological Associates, Bethesda, Maryland). The parasites were cloned by limiting serial dilution and then stabilized by a modification of the method of Lumsden et al. (1973).

### Infection procedure and monitoring of parasitemia

In preparation for infection, stabulates were passaged through naive mice. On day 6 or 7 of the infection the parasites were isolated and resuspended in cold PBS at a concentration of  $5 \times 10^4$  organisms/ml. Control and experimental mice were inoculated i.p. with 0.2 ml of this suspension ( $1 \times 10^4$ /mouse), and the course of parasitemia was monitored by daily counts of tail-vein blood diluted in 0.84%  $\text{NH}_4\text{Cl}$  and examined on a Neubauer hemocytometer. Blood collection was performed at the same time each day to reduce variability due to circadian periodicity. Parasitemia was expressed as the number of parasites per millilitre of blood vs. day of infection.

### Preparation of monoclonal antibodies (MAb's)

To assist in the depletion studies, a panel of rat anti-mouse monoclonal antibodies specific for mouse T lymphocyte subsets was utilized. Hybridoma cell lines utilized included 30-H.12 (rat IgG<sub>2b</sub> specific for Thy-1.2 surface antigen found on all T lymphocytes), GK1.5 (rat IgG<sub>2b</sub> specific for the L3T4 surface antigen), and 53-6.72 [rat IgG<sub>2a</sub> specific for the Lyt-2 (cytotoxic/suppressor cell) antigen], which were obtained from ATCC. These antibodies are cytotoxic for target cells in the presence of complement (Ledbetter and Herzenberg, 1979). The GK1.5-produced MAb recognizes the L3T4 surface molecule associated with the T-helper ( $T_H$ ) lymphocyte (Dialynis et al., 1983) and is necessary for generation of T-dependent antibodies. Except for line 30-H.12, all hybridomas were adapted to serum-free culture medium (HB-101; Hana/Dupont, Boston, Massachusetts). The 30-H.12 cell line was adapted to HL-1 medium (Ventrex, Portland, Maine) containing 1% FBS. All hybridomas were maintained in high-density static phase suspension culture in Nunclon Cell Factories® (Hazelton, Lenexa, Kansas) for 5–7 days to facilitate secretion of MAb's. MAB-containing supernatant fluids were harvested by centrifugation, concentrated 100-fold by tangential flow ultrafiltration (Filtron, Clinton, Massachusetts), filter-sterilized, and adjusted to a protein concentration of 3 mg/ml with serum-free HB-101.

### Adoptive cell transfers

**Lymphocyte isolation:** Adoptive lymphocyte transfers were accomplished with cells from naive mice or animals fully recovered from a *T. musculi* infection (days 20–25 postinfection with no demonstrable parasitemia). These cells were enriched for T lymphocytes by a modification of the panning procedure of Mage

et al. (1977). Lymphocytes were isolated from single-cell splenocyte suspensions by differential centrifugation over Lympholyte-M gradients (Accurate Scientific, Westbury, New York). The cells were then washed and resuspended in RPMI-1640 medium (Cellgro, Herndon, Virginia) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO Life Technologies, Grand Island, New York), and plated in 75-mm<sup>2</sup> tissue culture flasks (Costar, Cambridge, Massachusetts) previously treated by overnight incubation with affinity-purified rat anti-mouse Ig antibody (Organon Teknika, Malvern, Pennsylvania). Following 2 45-min incubations at 4°C, the nonadherent cells (>95% Thy-1<sup>+</sup> by fluorescence-activated cell sorter analysis) were decanted and washed with PBS. Adherent cells (>90% Ig<sup>+</sup>) used in the B lymphocyte transfers were removed from the flasks by incubating the washed monolayers at 37°C for 1 hr in  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS, followed by vigorous pipetting with a Pasteur pipet directed at the monolayers. The B cells were then washed twice with PBS. B lymphocytes thus recovered were >95% viable. In experiments involving T lymphocyte subpopulation transfers, the cells were further purified by indirect panning for selected cell surface markers (Braun, 1985). Cells were separated into the T-helper and T-cytotoxic phenotypes by 2 protocols. In the first procedure, the T lymphocytes were incubated for 30 min on ice with MAB's specific for Lyt-1 ( $T_H$ ) or Lyt-2 ( $T_C$ ) surface markers and then panned on flasks coated with goat anti-rat Ig (Organon Teknika), followed by collection of the nonadherent populations for subsequent injection. In the second approach, the isolated T lymphocytes were incubated with concentrated GK1.5 antibody and panned on anti-rat Ig-coated flasks. The nonadherent population (L3T4<sup>+</sup>) was decanted, the adherent (L3T4<sup>+</sup>) population was collected by hydrostatic pressure as above, and both populations were washed twice in PBS and used for adoptive transfers. Cytofluorometric analysis revealed that this technique rendered populations consisting of >95% Lyt-1<sup>+</sup> (helper) or Lyt-2<sup>+</sup> (cytotoxic) cells, and >90% L3T4<sup>+</sup> and L3T4<sup>−</sup> cells, respectively.

To further examine the potential role of direct T lymphocyte-mediated cytotoxicity to the parasite, adoptive transfers were performed with lymphokine-activated killer (LAK) cells. It has been demonstrated (Merluzzi et al., 1984) that *in vitro* exposure of lymphocytes to high levels of interleukin 2 (IL-2) results in activation of the cells to nonspecific cytotoxicity for a variety of targets, including parasites. To examine the ability of these nonspecifically cytotoxic cells to effect direct parasite killing, a system was developed for induction of LAK cells. Splenocytes from naive mice were washed and resuspended at  $2 \times 10^6$  cells/ml in RPMI-1640 containing 0, 5, 25, 50, or 100 units/ml of purified human IL-2 (Electronucleonics, Silver Spring, Maryland; spec. act. 640 units/ml). The cultures were incubated at 37°C for 2–4 days, followed by extensive washing in PBS. The recovered cells were assessed for viability and then used as effector cells in the CTL assay (House et al., 1987) using the NK-resistant cell lines P815 and EL4 and the NK-susceptible cell line YAC-1. Following induction culture, LAK cells were injected intravenously (i.v.) into naive mice. The animals were allowed to rest overnight and were infected with *T. musculi*.

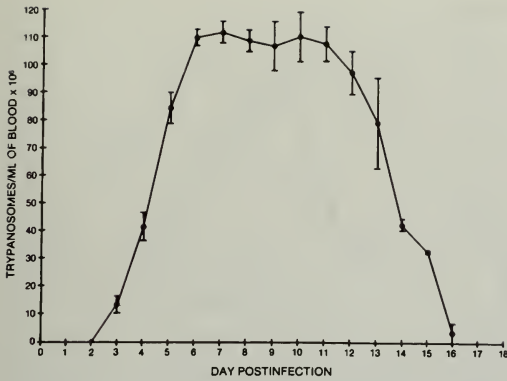


FIGURE 1. Course of *Trypanosoma musculi* infection in naive female B6C3F<sub>1</sub> mice. Values represent mean  $\pm$  SEM  $\times 10^6$  trypanosomes/ml of blood.

**Adoptive transfer procedure:** Cell transfers were performed by injecting isolated naive or immune lymphocytes i.v. into syngeneic recipient mice. Experimental design for transfers followed 4 protocols. In the first, naive mice received lymphocyte transfers from naive or immune donor mice. The animals were allowed to rest for 1 day, and were then infected with *T. musculi*. In the second protocol, mice were rendered T cell-deficient with a cocktail of MAb's 30-H.12 and GK1.5, were allowed to clear the antibody for 3 days, and were then reconstituted with transfer of T lymphocyte subpopulations from naive or immune donors. These mice were allowed to rest 1 day and were then infected with trypanosomes. In the third protocol, naive mice received various concentrations of naive T lymphocytes to assess the possible modulation of immunity by the presence of surplus T cells. Finally, mice received adoptive transfers of B lymphocytes from immune mice or mice in the late plateau phase of the infection to clarify the role of the B cell in parasite clearance.

#### Statistical analysis

Data analysis was performed by the Dunnett modification of Student's *t*-test for comparison of control to multitreatment groups. Data were considered significantly different from controls at  $P < 0.05$ .

## RESULTS

### Course of parasitemia in naive mice

The typical course of parasitemia observed in the B6C3F<sub>1</sub> mouse model was highly reproducible. There was a prepatent period of approximately 3 days followed by a logarithmic growth phase of parasites that peaked with approximately  $1.5 \times 10^8$  parasites/ml of blood on day 7 postinfection (Fig. 1). This was followed by a 4- or 5-day plateau phase with only mature (non-dividing) forms present in the blood, with an ensuing rapid clearance of parasites. Elimination of trypanosomes from the bloodstream was effected between days 15 and 17 postinfection (PI).

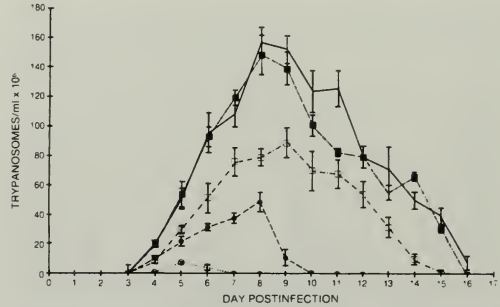


FIGURE 2. Kinetics of *Trypanosoma musculi* infection in mice following adoptive transfer of various concentrations of immune T lymphocytes. Values represent mean  $\pm$  SEM  $\times 10^6$  trypanosomes/ml of blood. Straight line signifies control values, open circles indicate mice transferred with  $5 \times 10^7$  immune T cells, closed circles indicate mice transferred with  $1 \times 10^7$  immune T cells, open squares indicate mice transferred with  $5 \times 10^6$  immune T cells, and closed squares indicate mice transferred with  $1 \times 10^6$  immune T cells. Results of a representative experiment, 5 mice per group.

### Adoptive lymphocyte transfer into naive mice

Transfer of unfractionated immune splenic T lymphocytes into naive mice altered the kinetics of a subsequent infection with trypanosomes in a dose-related manner (Fig. 2). Mice injected with  $1 \times 10^7$  immune T cells displayed a gradual increase of parasitemia, and once plateau phase was established on day 8 there was an almost immediate clearance of parasites from the blood. Mice receiving  $5 \times 10^7$  T lymphocytes demonstrated a reduced infection ( $< 5 \times 10^6$  organisms/ml) that terminated by day 7. Adoptive transfer of  $5 \times 10^6$  immune T cells resulted in greater than 45% decrease in plateau parasitemia. The basic kinetics of the infection was similar and the infection was eliminated on the same day in both groups.

Experiments were next performed to determine the time requirements for transfer. Mice were first infected with trypanosomes and then adoptively transferred with  $5 \times 10^7$  immune T lymphocytes on various days PI. As shown in Figure 3, transfer on the day of infection was protective, with only trace numbers of parasites present in the blood for 2 days. If the transfer was delayed until day 5 PI, protection was lost. However, once plateau was reached on day 7 there was an immediate elimination of the infection with complete clearance from the blood by day 9. Mice receiving the adoptive transfer on day 10 PI displayed no discernable difference from control animals.



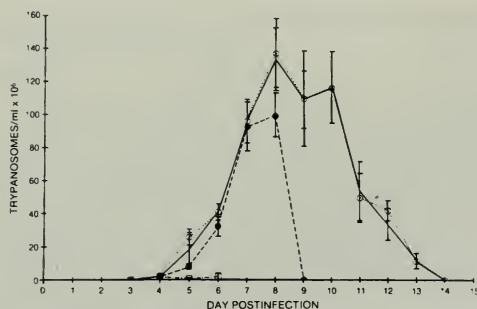


FIGURE 3. Kinetics of *Trypanosoma musculi* infection in mice following adoptive transfer of  $5 \times 10^7$  immune T lymphocytes at various days of infection. Values represent mean  $\pm$  SEM  $\times 10^6$  trypanosomes/ml of blood. Straight line indicates control values, open circles indicate mice receiving transfer 10 days PI, closed circles indicate mice receiving transfer 5 days PI, and open squares indicate mice receiving transfer on day of infection (day 0). Results of a representative experiment, 6 mice per group.

**Transfer of naive T lymphocytes:** Adoptive transfer of naive T lymphocytes was performed to determine whether alteration in infection kinetics was specific for immune cells or due only to an increased number of lymphocytes present in the host. Transfer of  $1 \times 10^7$  or  $5 \times 10^7$  naive T lymphocytes into naive mice resulted in enhancement of parasitemia levels (data not shown). The prepatent phases were identical and the infection was eliminated from the bloodstream on the same day (day 14) in both control and experimental mice. Enhanced parasitemia was most apparent during the plateau phase of the infection and might result from the increased number of suppressor cells resulting from the adoptive transfer. Transfer with fewer than  $1 \times 10^7$  naive T cells had no effect on the infection as measured by level of parasitemia (data not shown).

**Adoptive transfer of T lymphocyte subpopulations:** Immunocompetent mice receiving adoptive transfers with immune cytotoxic/suppressor (Lyt-2<sup>+</sup>) cells displayed a normal course of infection up until the initiation of plateau. This was followed by an accelerated clearance of the infection, terminating on day 12 of infection, compared to day 16 for control mice (Fig. 4a). Transfer with immune T lymphocytes of the Lyt-1<sup>+</sup> phenotype led to an abbreviated infection, with a peak parasitemia of  $6.4 \times 10^7$  trypanosomes/ml of blood (vs.  $1.7 \times 10^8$ /ml in controls) and clearance from the blood by day 8 PI.

As a corollary experiment to those described immediately above, mice were adoptively transferred with immune T lymphocytes positive or

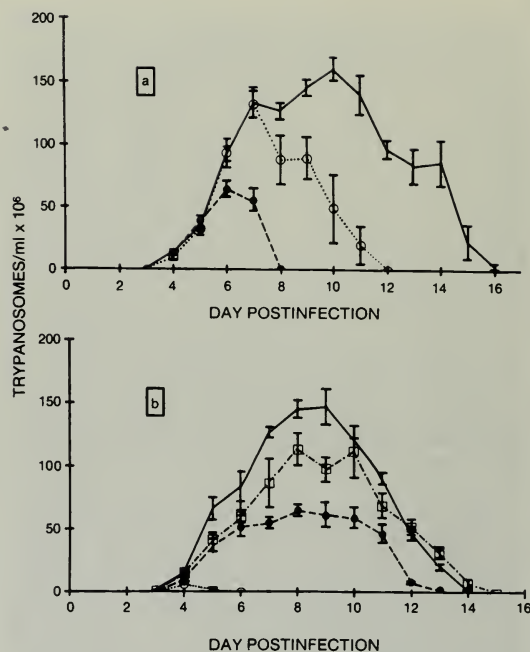


FIGURE 4. a. Kinetics of *Trypanosoma musculi* infection following adoptive transfer of 1 animal equivalent of Lyt-1<sup>+</sup> or Lyt-2<sup>+</sup> T lymphocytes. Values represent mean  $\pm$  SEM trypanosomes/ml of blood. Straight line indicates controls, open circles indicate Lyt-2<sup>+</sup> T cells, and closed circles indicate Lyt-1<sup>+</sup> T cells. Results of a typical experiment, 6 mice per group. b. Kinetics of *Trypanosoma musculi* infection following adoptive transfer of 1 animal equivalent number of L3T4<sup>+</sup> or L3T4<sup>-</sup> T lymphocytes. Values represent mean  $\pm$  SEM trypanosomes/ml of blood. Straight line indicates control values, open circles indicate unfractionated T cells, closed circles indicate L3T4<sup>+</sup> T cells, and open squares indicate L3T4<sup>-</sup> T cells. Results of a representative experiment, 5 mice per group.

negative for the L3T4 surface antigen (i.e., helper cell phenotype). Although the kinetics of infection for all groups were basically similar, there were differences in the level of parasitemia achieved (Fig. 4b). Mice receiving L3T4<sup>-</sup> lymphocytes displayed a slight, but significant, reduction in parasitemia during the plateau phase, although the levels were similar during the log increase phase and during elimination. In contrast, transfer of L3T4<sup>+</sup> cells resulted in up to a 64% lower blood level of *T. musculi* as compared to control mice. As a positive control, an unfractionated population of immune T lymphocytes was included in this experiment. As can be seen, adoptive transfer of this population resulted in only a transient expression of parasites in the circulation that was resolved within 1 day of infection (Fig. 4b).

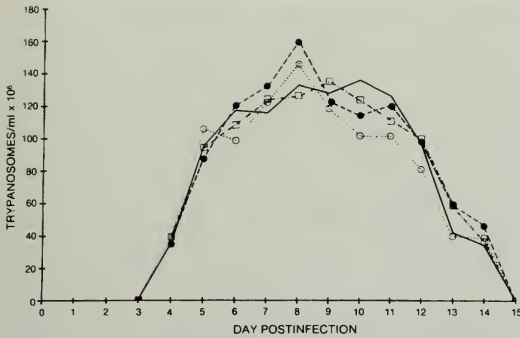


FIGURE 5. Kinetics of *Trypanosoma musculi* infection following adoptive transfer with various concentrations of lymphokine-activated killer (LAK) cells. Values represent mean  $\pm$  SEM trypanosomes/ml of blood. Straight line indicates controls, open circles indicate  $1 \times 10^7$  LAK cells, closed circles indicate  $5 \times 10^6$  LAK cells, and open squares indicate  $1 \times 10^6$  LAK cells. Results of a representative experiment, five mice per group.

**Transfer of lymphokine-activated killer cells:** Lymphokine-activated killer cells have been shown to possess the  $\text{Lyt-1}^-$ ,  $\text{Lyt-2,3}^+$  phenotype and to nonspecifically lyse a variety of NK-resistant tumor cell lines. There is also some evidence that LAK cells may play a role in host resistance to certain parasites. As seen in Table I, cultures not supplemented with IL-2 displayed only background cytotoxicity toward the tumor line P815 ( $<5\%$ ), whereas splenocytes cultured with IL-2 lysed P815 target cells in a dose-responsive manner up to approximately 60%. Similar results were seen with 2 other cell lines tested (data not shown). Peak activity of LAK was realized on day 4 of culture, with an optimal dose of between 50 and 100 units/ml of IL-2. To avoid the possibility of activating suppressor cells, 50 units/ml of IL-2 was used in all subsequent cultures.

Once LAK cell production was optimized, these cells were adoptively transferred into naive mice to assess their *in vivo* efficacy in killing parasites. It was found that transfer of up to  $1 \times 10^7$  LAK cells had no significant effect on the course of *T. musculi* infection in mice (Fig. 5), although duplicate cultures were up to 70% cytotoxic for various tumor cell lines.

**Effect of B lymphocyte transfers:** Adoptive transfer of late plateau-phase (day 10 PI) B lymphocytes into naive mice resulted in an enhanced elimination of the parasites from the blood. Transfer of  $5 \times 10^7$  B cells resulted in an infection with barely detectable numbers of parasites in the blood (Fig. 6a), and elimination occurred 3

TABLE I. In vitro induction of murine lymphokine-activated killer (LAK) cells with purified human interleukin 2.\*

IL-2 concentration	% Cytotoxicity†		
	25:1	12:1	6:1
Control	$6.0 \pm 2.4$	$2.4 \pm 2.6$	$1.9 \pm 2.0$
5 units/ml	$19.2 \pm 1.1\ddagger$	$8.3 \pm 0.6\ddagger$	$7.2 \pm 0.8\ddagger$
25 units/ml	$31.3 \pm 0.8\ddagger$	$23.2 \pm 1.5\ddagger$	$14.6 \pm 1.2\ddagger$
50 units/ml	$55.3 \pm 2.0\ddagger$	$38.2 \pm 2.4\ddagger$	$35.0 \pm 4.2\ddagger$
100 units/ml	$56.5 \pm 3.5\ddagger$	$39.7 \pm 1.7\ddagger$	$34.4 \pm 5.5\ddagger$

\* Induction of LAK cells was performed with  $2 \times 10^6$  naive murine splenocytes cultured with various concentrations of purified human IL-2 in a 4-day induction culture.

† Percent nonspecific cytotoxicity  $\pm$  SEM against P815 mastocytoma tumor targets as assessed in a 4-hr  $^{51}\text{Cr}$ -release assay. Cytotoxicity was measured at 3 effector:target ratios.

‡ Significantly different from respective controls at  $P < 0.05$ .

days earlier than in control mice. Transfer of  $1 \times 10^7$  B cells resulted in approximately a 50% reduction in parasitemia, although the course of the infection and its eventual clearance were not significantly different from that of control animals.

The adoptive transfer of  $>$ day 20 immune B cells into naive mice resulted in a dose-related down-modulation of the subsequent infection (Fig. 6b). Transfer of  $5 \times 10^7$  immune B lymphocytes reduced the infection to only 2 days duration, with a maximum of  $<8 \times 10^6$  parasites/ml of blood on day 5 of infection, and termination of the infection on day 7. Transfer of  $1 \times 10^7$  lymphocytes resulted in a 60% reduction in parasitemia, which peaked on day 6 rather than day 7 as in controls. This peak was followed by a rapid elimination of parasites by day 9 of infection. With both  $1 \times 10^6$  and  $5 \times 10^6$  transferred cells, the major effect was manifested as a shift in day of clearance following establishment of the plateau phase on day 7 of infection (2 days early and 4 days early, respectively). The ability to modulate the infection kinetics was seen with as little as  $1 \times 10^6$  transferred immune B lymphocytes.

**Reconstitution of T lymphocyte-depleted mice:** To confirm previous observations from MAb-mediated cell abrogations and adoptive transfers, the 2 approaches were combined for adoptive transfer into T lymphocyte-deficient mice. In these experiments mice were treated with a MAb cocktail containing both anti-Thy-1 and anti-L3T4, followed by reconstitution through adoptive lymphocyte transfer. *In vivo* treatment with this MAb cocktail was found to render mice functionally T cell deficient (Table II). Because



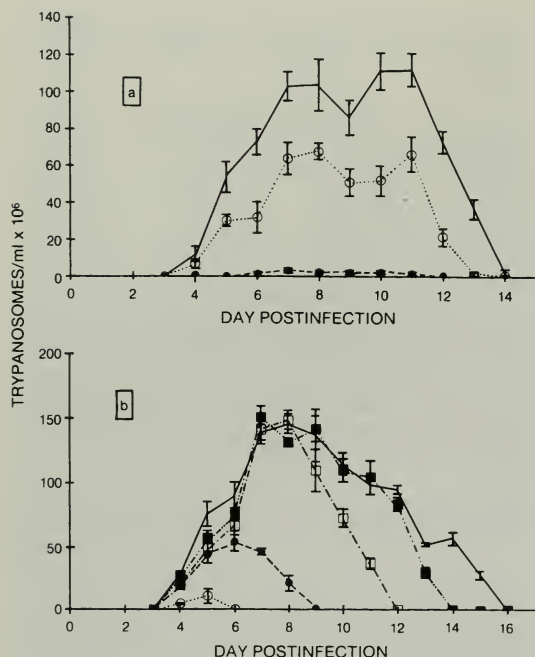


FIGURE 6. a. Kinetics of *Trypanosoma musclicoli* infection following adoptive transfer with B lymphocytes collected from plateau-phase mice. Values represent mean  $\pm$  SEM trypanosomes/ml of blood. Straight line indicates control values, open circles indicate  $1 \times 10^7$  B cells, and closed circles indicate  $5 \times 10^7$  B cells. Results of a typical experiment, 5 mice per group. b. Kinetics of *Trypanosoma musclicoli* infection following adoptive transfer with B lymphocytes collected from mice following elimination of parasite from the blood. Values represent mean  $\pm$  SEM trypanosomes/ml of blood. Straight line indicates control values, open circles indicate  $5 \times 10^7$  B cells, closed circles indicate  $1 \times 10^7$  B cells, open squares indicate  $5 \times 10^6$  B cells, and closed squares indicate  $1 \times 10^6$  B cells. Results of a representative experiment, 5 mice per group.

the previous studies implicated L3T4<sup>+</sup> cells as responsible for T-dependency in this model, the adoptive transfer studies utilized L3T4<sup>-</sup> or L3T4<sup>+</sup> lymphocytes from either naive or immune mice. L3T4<sup>-</sup> T lymphocytes had no effect on the characteristic immunosuppression produced by *in vivo* MAb treatment (Fig. 7), consistent with the results of transfer experiments in immunocompetent mice. Transfer of L3T4<sup>+</sup> cells from naive mice resulted in an infection that was only slightly different from L3T4<sup>-</sup> mice, although still significantly elevated above control values. However, transfer of L3T4<sup>+</sup> lymphocytes from immune mice resulted in a greater than 50% reduction in peak parasitemia. This agreed with earlier observations following adoptive transfer

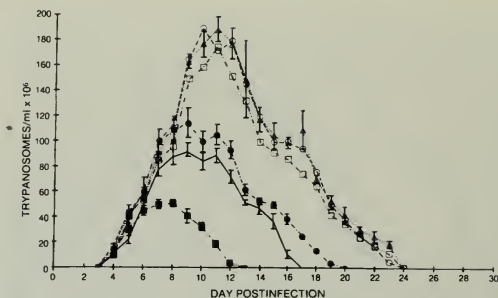


FIGURE 7. Kinetics of *Trypanosoma musclicoli* infection following adoptive transfer of immune or naive T-helper (L3T4<sup>+</sup>) or T-cytotoxic (L3T4<sup>-</sup>) lymphocytes into T-deficient mice. Values represent mean  $\pm$  SEM trypanosomes/ml of blood. Straight line indicates control values, triangles represent mice following *in vivo* treatment with anti-T lymphocyte monoclonal antibodies (T-deficient mice), open circles represent T-deficient mice adoptively transferred with naive T-cytotoxic cells, closed circles represent T-deficient mice adoptively transferred with naive T-helper cells, open squares represent T-deficient mice adoptively transferred with immune T-cytotoxic cells, and closed squares represent T-deficient mice adoptively transferred with immune T-helper lymphocytes. Results of a representative experiment, 5 mice per group.

into normal mice. Unlike the data presented earlier, transfer of L3T4<sup>+</sup> cells in T-deficient mice resulted in a significantly reduced duration of infection (3 days shorter).

## DISCUSSION

The elimination of *Trypanosoma musclicoli* from the bloodstream of the mouse is known to require the presence of functional T lymphocytes (Viens, 1985). Previous reports (Viens et al., 1975; Pouliot et al., 1977; Targett et al., 1981) had failed to fully characterize the nature of this T-dependency in detail, primarily due to a lack of appropriate reagents or methodology. In a recent study by our laboratory utilizing selective *in vivo* immunodepletion studies (House and Dean, 1988), it was demonstrated that this T-dependency of parasite clearance is mediated by the T-helper lymphocyte subset. Further, it was shown that the Lyt-2<sup>+</sup> T-cytotoxic cell plays little apparent role in induction of immunity to the organism. The purpose of the study described here was to further examine this T-dependency by the use of adoptive cell transfer techniques.

Adoptive cellular transfers have been utilized in numerous studies to examine the nature of immunity to parasitic diseases, including *Trypanosoma musclicoli* (Takayanagi and Naka-

take, 1976; Targett et al., 1981; Lima et al., 1984; Meeusen et al., 1984). To date, these studies have used poorly characterized, highly heterogeneous cell populations, including thymus glands, peritoneal exudate cells, etc. The advent of monoclonal antibody reagents has provided researchers in immunoparasitology with a unique tool with which to study the relationship of various cell subsets in host/parasite relationships (McBride, 1983). In particular, the use of monoclonal antibodies specific for lymphocyte subsets may be combined with lymphocyte panning (Mage et al., 1977; Braun, 1985) for preparative scale separation of lymphocytes. This facilitates the preparation of large quantities of adoptive cells at a level of purity previously unattainable.

The adoptive transfer of immune splenic T lymphocytes into naive mice was found to modulate a subsequent infection with *T. muscui* in direct proportion to the number of adoptive cells transferred. This modulation was manifested as a reduction in parasitemia and kinetics of infection, with adoptive transfer of  $5 \times 10^7$  immune cells/mouse effectively preventing the establishment of patent infection. This protective feature was noted only if transfer occurred before patency of infection. If the transfer was delayed until patency, no effect was seen until establishment of the plateau phase, when there was an immediate clearance of parasites from the blood. Transfer of cells during the plateau phase had no effect on the subsequent course of the infection, suggesting that the transferred cells altered later-acting immune mechanisms (e.g., induction of parasite-specific antibodies), rather than causing direct toxicity to the parasites. Transfer of naive T lymphocytes had no significant effect on parasitemia, indicating that the effects seen with immune T lymphocytes were specific and not due to the presence of increased numbers of lymphoid cells.

Immune T lymphocyte preparations were next subdivided into 2 major groups for transfer, the T-helper phenotype (Lyt-1<sup>+</sup>/L3T4<sup>+</sup>) and the T-cytotoxic/suppressor phenotype (Lyt-2<sup>+</sup>/L3T4<sup>-</sup>). In general, it was found that adoptive transfer with Lyt-1<sup>+</sup> cells resulted in a down-modulation of infection similar to that seen with whole-population T lymphocytes. However, these experiments also demonstrated a significant alteration in infection following transfer with T-cytotoxic cells. This finding was in conflict with our previously stated hypothesis (House and Dean, in press) that the T-cytotoxic cell plays no part

TABLE II. *Functional status of murine splenic lymphocytes following in vivo administration of anti-subset monoclonal antibodies.*

Treatment	CTL function*	PFC response†
Control	75.0	1,760 ± 59
Anti-Thy-1.2	ND	240 ± 59‡ (-86%)
Anti-L3T4 (T-helper)	38.4‡ (-49%)	0.0 ± 0‡ (-100%)
Anti-Lyt-2 (T-cytotoxic/suppressor)	29.1‡ (-61%)	2,610 ± 160‡ (+48%)
Anti-Thy-1/L3T4	5.0‡ (-93%)	0.0 ± 0‡ (-100%)

\* Values represent mean specific cytotoxicity of P815 mastocytoma target cells in a 4-hr lysis assay. Results of a typical experiment, 5 mice per group. ND = not determined.

† Values represent mean ± SEM plaque-forming cells per 10<sup>6</sup> splenocytes. Results of a typical experiment, 6 mice per group.

‡ Significantly different from control values at  $P < 0.05$ .

in clearance of *T. muscui*. This discrepancy is probably due to the fact that cells negatively selected by anti-Ig panning were invariably contaminated with a small number of the complementary phenotype. Therefore, in populations of T-cytotoxic transfer cells, the contaminating Lyt-1<sup>+</sup> T-helper cells could, upon contact with specific parasite antigen, undergo clonal expansion, and thereby obfuscate the findings. More definitive results were obtained with transfer of L3T4 positive or negative cells. In these experiments, transfer of L3T4<sup>+</sup> cells resulted in a significant reduction of parasitemia, whereas transfer with L3T4<sup>-</sup> cells resulted in kinetics of infection more similar to control values. These data provide further evidence that the T-cytotoxic cell does not play an important role in immunity to *T. muscui*.

Adoptive transfer with lymphokine-activated killer cells had no effect on trypanosome infection. The LAK induction protocol developed and utilized here resulted in a population of Thy-1/Lyt-2<sup>+</sup> lymphocytes activated for nonspecific cytotoxic capacity. Whereas these LAK cells were not classical cytotoxic T lymphocyte-type cells, they were found to be capable of potent cytolytic activity (approximately 60%). A potential role for the T-helper lymphocyte in clearance of parasitemia would conceivably be the activation of nonspecific cytolytic effector cells by T-helper-derived lymphokines. As stated, there was no effect following adoptive transfer of these cells. These studies provide strong evidence that clearance of *T. muscui* from mice is not mediated by Lyt-2<sup>+</sup> lymphocytes.



Recent studies (Wechsler and Kongshavn, 1985, 1986) have demonstrated the induction of *T. musculi*-specific antibodies and characterized their role in elimination of the infection. In addition, it has been shown (Vargas et al., 1984) that selective depletion of B lymphocytes from mice results in complete inability of the animals to recover from a *T. musculi* infection. In the current study, adoptive transfer of immune (but not naive) B lymphocytes resulted in a change in parasitemia similar to that seen following immune T lymphocyte transfer. B lymphocytes from animals in the plateau phase of infection were capable of reducing the infection, although they were not as effective as cells from mice having cleared the parasites from the blood. This is reflected in the kinetics of infection, where the rapid clearance of parasites from the blood following the plateau phase may be due to a critical level of antibody-producing cells.

In a confirmatory experiment, mice were rendered T lymphocyte-deficient by administration of MAb's followed by adoptive transfer with naive or immune L3T4<sup>+</sup> or L3T4<sup>-</sup> lymphocytes. From the results it is apparent that the L3T4<sup>-</sup> cells were unable to reconstitute the ability of mice to eliminate the infection. In contrast, the transfer of naive L3T4<sup>+</sup> cells into these T-deficient mice resulted in a course of infection essentially identical to control animals, and transfer of immune L3T4<sup>+</sup> cells resulted in significantly lower parasitemia and abbreviated duration of infection compared to respective controls.

In conclusion, the results presented here provide evidence that T lymphocyte-dependency of *Trypanosoma musculi* clearance from the blood of the mouse requires the T-helper lymphocyte, possibly for the induction of specific antibody. Further, our studies imply a lack of direct cytotoxicity to the parasite by T lymphocytes or related effectors (i.e., LAK cells). Although trypanosome-specific antibodies are known to be necessary for the clearance, the exact mechanism of clearance remains unknown. For example, whereas antibody, complement, macrophages, and platelets have all been implicated as playing a role in *T. musculi* clearance (Viens, 1985), a model of their interrelationship has not been established and should provide the basis for future experiments.

#### ACKNOWLEDGMENTS

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## IDENTIFICATION OF ANTIGENS OF *SARCOCYSTIS CRUZI* SPOROZOITES, MEROZOITES, AND BRADYZOITES WITH MONOCLONAL ANTIBODIES

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**ABSTRACT:** Sporozoites and culture-derived merozoites of *Sarcocystis cruzi* were used to elicit monoclonal antibodies (MAB's) in mice. Some of these antibodies reacted with the surface of live sporozoites and merozoites as determined by immunofluorescence. An array of similar antigens was identified in Western blots of sporozoites by both anti-merozoite MAB's and an anti-sporozoite MAB. At least 1 antigen in blots of bradyzoites was identified by anti-merozoite MAB's and a cluster of antigens was identified by an anti-sporozoite antibody. These results indicate that several surface epitopes of sporozoites and merozoites are shared with molecules of bradyzoites and that antigen patterns of molecules bearing these epitopes in 3 stages of *Sarcocystis* may be either distinct or similar.

*Sarcocystis cruzi* is a 2-host coccidian parasite that uses domestic cattle as intermediate hosts and carnivores, such as the coyote and domestic dog, as definitive hosts (Dubey, 1976). In the definitive host, bradyzoites of *S. cruzi* develop in the gut to gametocytes and then oocysts that quickly sporulate and are shed as oocysts or sporocysts in the feces. After ingestion by the bovine host, sporozoites excyst from sporocysts, invade gut tissue, and presumably travel via the circulation to small to medium-sized muscular arteries (i.e., mesenteric arteries) where they enter endothelial cells and develop by merogony (schizogony) to produce merozoites (Dubey, 1982). After several merogonous generations, merozoites enter various muscle cells where the parasite multiplies by endodyogeny, eventually forming bradyzoites that are infective to the definitive host.

Although *S. cruzi* is an economically important parasite of domestic cattle (Fayer and Dubey, 1986) little is known about the bovine immune response to *S. cruzi* or about the antigenic make-up of the various morphological stages (i.e., sporozoite, merozoite, and bradyzoite). Recently we examined the reactions of sera from calves infected with *S. cruzi* (Speer et al., unpubl.). To initiate studies designed to clarify the nature of the bovine immune response to *S. cruzi* and compare antigen components of the 3 major stages of *S. cruzi*, we prepared monoclonal antibodies (MAB's) to sporozoites of *S. cruzi* from

sporocysts and to merozoites produced by *in vitro* cultivation as recently described (Speer and Dubey, 1986; Speer et al., 1986a, 1986b). This paper reports our findings from experiments regarding the molecular nature of antigenic determinants defined by some of these anti-*Sarcocystis* MAB's on sporozoites, merozoites, and bradyzoites of *S. cruzi*.

### MATERIALS AND METHODS

#### Parasite production

Sporozoites of *S. cruzi* were obtained from sporocysts kindly provided by Dr. J. P. Dubey (Zoonotic Diseases Laboratory, Livestock and Poultry Science Institute, U.S.D.A., Beltsville, Maryland), sporulated, and purified as previously described (Speer et al., 1986a). Bradyzoites were purified from muscle of experimentally infected bovine calves and were also obtained from Dr. Dubey. Merozoites of *S. cruzi* were produced *in vitro* by infection of bovine cardiopulmonary artery endothelial cells (CPA; American Type Culture Collection, Rockville, Maryland) as described previously (Speer and Dubey, 1986; Speer et al., 1986a, 1986b).

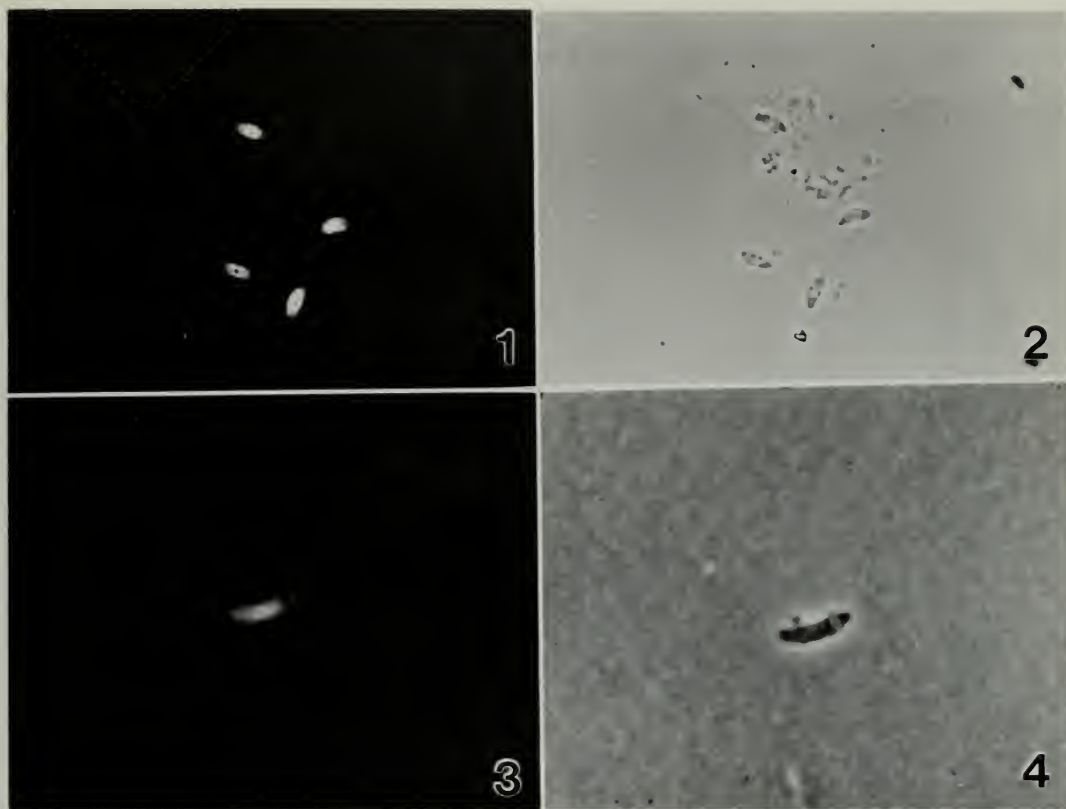
#### Antigen preparations

Sporozoites and merozoites were pelleted by centrifugation (400 g, 10 min, 20 C), washed 2× in Dulbecco's modified phosphate-buffered saline (GIBCO, Grand Island, New York) by centrifugation, and either used directly or stored at -70 C. Bradyzoites were solubilized directly in treatment buffer (see below) or stored at -70 C.

Bradyzoites were obtained from the digests of muscles of a calf experimentally infected with the Be-1 isolate of *Sarcocystis cruzi* (Fayer and Dubey, 1984). Muscles that contained sarcocysts were ground in a meat grinder and then incubated at 37 C for 15 min in acid-pepsin solution (Lunde and Fayer, 1977). After filtering through 2 layers of cheesecloth, the digest was centrifuged in 50-ml tubes at 400 g for 10 min, and the pellet was resuspended in Hanks' balanced salt solution (HBSS), pH 7.2. This suspension was centrifuged at 400 g for 10 min, decanted, and the upper brown

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FIGURES 1-4. Reaction of MAB's with *S. cruzi* merozoites from CPA culture. 1. Fluorescence photomicrograph of acetone-fixed merozoites treated with MAB 31.1B1.1 and fluorescein-labeled goat anti-mouse IgG (H&L).  $\times 560$ . 2. Phase-contrast photomicrograph of the same field as Figure 1.  $\times 560$ . 3. Fluorescence photomicrograph of live merozoite reacted with MAB 31.1B1.1.  $\times 1,760$ . 4. Phase-contrast photomicrograph of the same field as Figure 3.  $\times 1,760$ .

layer of muscle fibers was removed leaving white bradyzoites in the pellet. Bradyzoites from several tubes were pooled, centrifuged, resuspended in HBSS, re-centrifuged, and the supernatant discarded. The pellet of zoites was mixed with about 10 ml of 50% isotonic Percoll solution in a conical tube and centrifuged at 400 g for 10 min. Clean zoites were collected from the bottom of the centrifuge tubes, washed in HBSS to remove Percoll, resuspended in HBSS, and stored at  $-70^{\circ}\text{C}$ .

#### Monoclonal antibody production

Monoclonal antibodies (MAB's) were prepared by immunization of female BALB/cBy mice, 8-10 wk of age, with  $1 \times 10^6$  merozoites or sporozoites per mouse mixed 1:1 (v/v) in complete Freund's adjuvant by intraperitoneal injection. One month later mice were given  $8 \times 10^5$  merozoites or  $5 \times 10^5$  sporozoites, respectively, by intravenous injection, and 3 days later the spleens were removed and fused to the myeloma cell line P3/X63-Ag8.6.5.3 (Kearney et al., 1979) and selected by previously described methods (Galfre et al., 1977). When growth of primary hybridoma cultures was apparent (about 14 days) hybridoma culture supernatants were screened by an indirect immunofluorescence assay (IFA) as previously described (Burgess et al., 1984; Burgess, 1986), employing acetone-fixed slide preparations of merozoites and sporozoites. Positive hybridoma cultures were selected, cloned by limiting dilution, retested, and expanded in culture. Supernatant culture fluids were concentrated  $10\times$  by vacuum dialysis, stored at  $-70^{\circ}\text{C}$ , and used for all experiments described unless otherwise indicated.

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#### Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and live IFA

Antigen analysis by SDS-PAGE, Western blotting, and live IFA were essentially as described (Burgess and Jerrells, 1985; Burgess, 1986) except that merozoites, sporozoites, or bradyzoites were electrophoresed on 0.75-mm-thick 8-cm slab gels (12% T) at 200 V constant voltage for 1.5 hr (Hoefer Scientific Instruments, San Francisco, California) and then blotted onto nitrocellulose for 2 hr at 70 V, 10 C (Transblot system, BioRad, Richmond, California). Antigens were detected employing horseradish peroxidase (HRP) anti-mouse IgG + IgM + IgA (H&L) (Cooper Biomedical/Cappel Laboratories, Malvern, Pennsylvania) as pre-



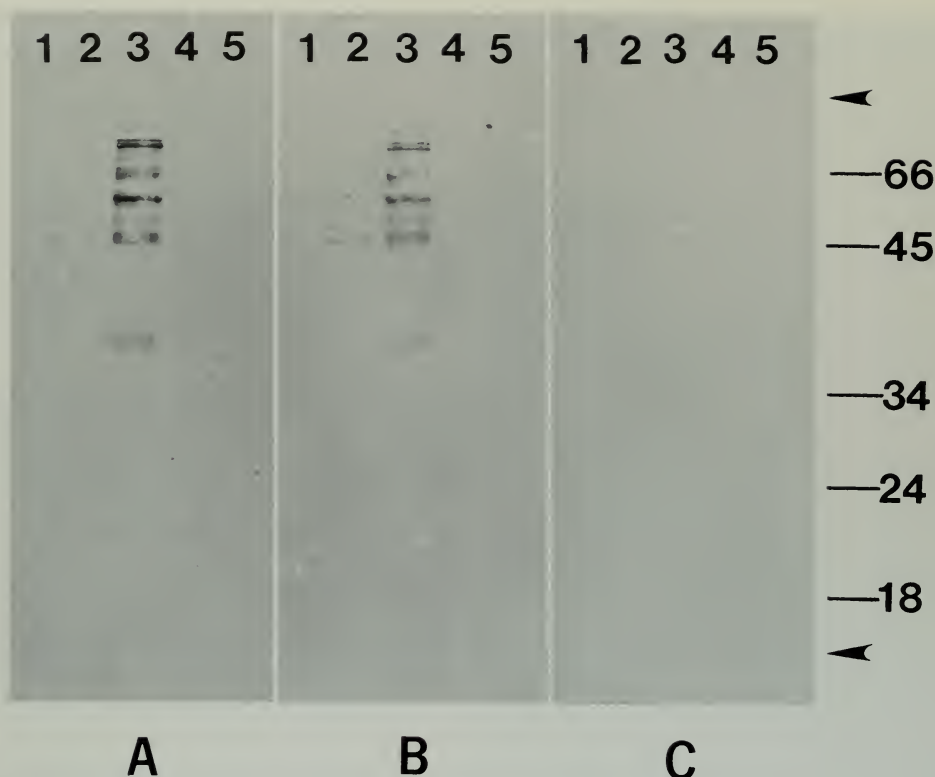


FIGURE 5. Western blot analysis of *S. cruzi* antigens with 2 MAB's (panels A, B) and a control myeloma supernatant (panel C). MAB's: A, 31.2D1.3, B, 31.1B1.1. Antigens: lane 1, bovine muscle; lane 2, bradyzoites; lane 3, sporozoites; lane 4, CPA culture-derived merozoites; lane 5, CPA cells. The relative migration of molecular weight standards ( $\times 1,000$ ) is indicated to the right (66, bovine serum albumin; 45, ovalbumin; 34, pepsin; 24, trypsinogen; 18,  $\beta$ -lactoglobulin; 14, lysozyme).

viously described (Burgess and Jerrells, 1985; Burgess, 1986).

### RESULTS

Several MAB's elicited against *S. cruzi* merozoites reacted with acetone-fixed merozoites and showed uniform fluorescence patterns on merozoites from CPA cultures (Fig. 1). When live merozoites were exposed to anti-merozoite MAB's in the live IFA, a uniform surface fluorescence was apparent (e.g., 31.1B1.1, Fig. 3). The anti-sporozoite MAB 15.5A5.1 also bound to the surface of live sporozoites showing a similar uniform fluorescence pattern (data not shown).

Western blot analysis revealed limited MAB reactivities as well as shared epitopes among stages of *S. cruzi*. Several surface-reactive MAB's were identified by live IFA and antigens from eliciting stages were detected on Western blots. For example, the anti-merozoite MAB's 31.2D1.3 and 31.1B1.1 reacted with bradyzoite and spo-

rozoite antigens but not with merozoite antigen (Fig. 5A, B). Alternatively, the anti-sporozoite MAB 15.5A5.1 identified several antigens in bradyzoite, sporozoite, and merozoite preparations (Table I; Fig. 6). The relative molecular weights (MW) of the antigens identified in this study are summarized in Table I. These data (Figs. 5, 6; Table I) show that the anti-sporozoite MAB 15.5A5.1 and the anti-merozoite MAB 31.1B1.1, both surface-reactive by the IFA, identified virtually identical bands in sporozoites (compare lane 3, Fig. 5B to lane 3, Fig. 6). In addition, MAB 15.5A5.1 reacted with epitopes on groups of polypeptides in merozoites and bradyzoites (Table I). The anti-merozoite MAB's did not react with merozoites after Western blotting (Table I), although all were reactive with a 45,500 MW bradyzoite antigen and in the IFA. One anti-merozoite MAB 31.1B1.1 was reactive with merozoites in the live IFA (Fig. 3). Further Western blot analysis with the anti-sporozoite MAB 15.5A1.3 resulted in no detectable reaction with

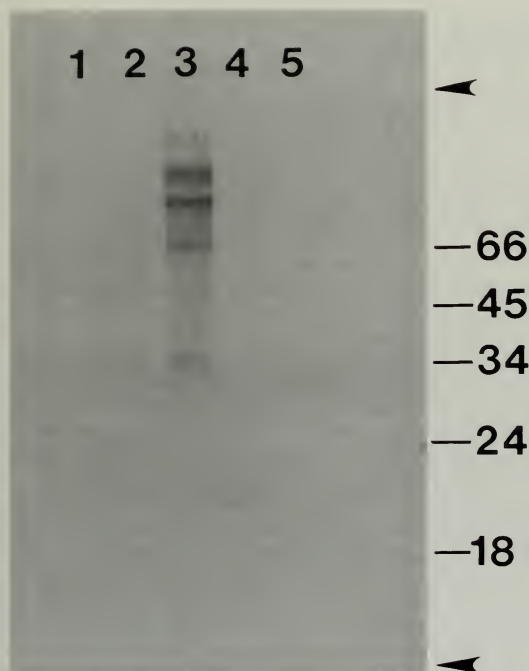


FIGURE 6. Western blot analysis of *S. cruzi* antigens with the anti-sporozoite MAb 15.5A5.1. Antigens and molecular weight standards ( $\times 1,000$ ) were as in Figure 5.

bradyzoite, sporozoite, or merozoite antigens (data not shown). Thus, several antigens were identified over a range of molecular weights with the exception of a single polypeptide (approx. 45,500 MW) for bradyzoites probed with the anti-merozoite MAb's (Fig. 5A, B; Table I).

## DISCUSSION

Three morphological forms of *Sarcocystis cruzi* have been examined by reaction with MAb's

directed against sporozoites from sporocysts and merozoites produced in an *in vitro* culture system (Speer and Dubey, 1986; Speer et al., 1986a, 1986b). Although 1 anti-merozoite MAb reacted with the surface of merozoites (Fig. 3), none identified antigens in Western blots of merozoites (Fig. 5; Table I). This result could be due to denaturation of epitopes during the SDS-PAGE procedure or to heat lability above 30 C as has been recently reported for *Rickettsia conorii* (Dasch et al., 1987, Abstract, 87th Annual Meeting of the American Society for Microbiology, Atlanta, Georgia). Extreme sensitivity to endogenous proteases could have also caused destruction of epitopes on surface antigens.

Two anti-sporozoite MAb's reacted with the surface of sporozoites and 1 (15.5A5.1) reacted with antigens in Western blots of merozoites, bradyzoites, and sporozoites (Fig. 6; Table I). One MAb (15.5A1.3) that reacted with the sporozoite surface did not react with any antigens in any of the stages tested by western blotting (Table I).

Both complex and single antigen bands were detected with MAb's, however. For example, a single 45,500 MW band in bradyzoites was detected with surface-reactive and nonsurface-reactive anti-merozoite MAb's (Table I). The same MAb's reacted with at least 6 bands in sporozoites with molecular weights ranging from  $<18,000$  to  $>60,000$  (Fig. 5). Thus, a 45,500 MW band in both bradyzoites and sporozoites bears similar epitopes not detected in blots of merozoites (Table I). An anti-sporozoite MAb 15.5A5.1 identified epitopes shared by merozoites and bradyzoites (Table I) as well as an antigen array in sporozoites similar to the array identified in sporozoites by 3 anti-merozoite MAb's (31.1B1.1, 31.2D1.2, and 31.2D1.3, Ta-

TABLE I. Summary of antigen analysis with monoclonal antibodies elicited against *Sarcocystis cruzi*.

MAb/isotype	Live IFA*	Western blot results†		
		Merozoite	Bradyzoite	Sporozoite
Anti-sporozoite				
15.5A1.3/IgM	+			
15.5A5.1/IgM	+	>60, 65, 55	45.5, 64	<18, 34, 46, 64, >60
Anti-merozoite				
31.1B1.1/IgG <sub>2a</sub>	+		45.5	<18, 34, 46, 64, >60
31.2D1.2/IgM			45.5	<18, 34, 46, 64, >60
31.2D1.3/IgM			45.5	<18, 34, 46, 64, >60

\* Results are given for reaction on eliciting stage.

† Reactions are given for whole antigen preparations of the indicated stage and as relative molecular weights ( $\times 1,000$ ). Blank spaces indicate no reaction detected.



ble I). These results illustrate that antigens detectable on the surface of live merozoites and sporozoites share epitopes with antigens found in bradyzoites and that these epitopes may be on single or multiple polypeptides.

Several polypeptide bands of similar molecular weight have recently been identified by SDS-PAGE of *S. cruzi* merozoites, bradyzoites, and sporozoites (Speer et al., unpubl.). For example, bands at 35,000, 43,000, and 44,000 MW were found in both merozoites and sporozoites, whereas sporozoites and merozoites had 49,000 and 54,000 MW bands. Sera 88 days postinoculation from cows infected with *S. cruzi* sporocysts identified antigens of 33,000, 48,000, and 49,000 MW on Western blots of merozoites obtained from infected CPA cultures. Although some of these polypeptides could be related to the antigens identified by our MAb's (Fig. 5), further investigation will be needed to address this question.

Abbas and Powell (1983) have described several surface antigens of *S. muris* zoites taken from mouse muscle tissue identified by immunoprecipitation with rabbit antiserum to extracts of these stages. Prominent bands at 27,000 and 90,000 MW were precipitated by both rabbit antiserum and infected mouse sera, whereas an additional antigen of 43,000 MW was identified by the rabbit antiserum. We do not know at this point which, if any, of the molecules of *S. cruzi* identified by MAb's (Table I) or bovine antiserum (Speer et al., unpubl.) may be surface molecules, although some of the epitopes on antigens identified by MAb's are clearly on the surface of live *S. cruzi* merozoites and sporozoites.

#### ACKNOWLEDGMENTS

We thank Dr. J. P. Dubey for providing the sporocysts and bradyzoites of *S. cruzi* used in this work. We also thank Ms. Jean Kyle for her excellent technical assistance and Ms. Anne Angermeyer for preparation of the manuscript. This is paper number J 2096 of the Montana Agricultural Experiment Station.

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# THE SEXUAL STAGES OF *EIMERIA WYOMINGENSIS* HUIZINGA AND WINGER, 1942, IN EXPERIMENTALLY INFECTED CALVES

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**ABSTRACT:** Seven of 12 calves given  $10^6$  *Eimeria wyomingensis* sporulated oocysts had sexual stages of the parasite when examined at necropsy. Clinical signs of coccidiosis were not observed in any calf. Sexual stages were located in host cells in the lamina propria of the villi in the terminal small intestine. Infected host cells underwent nuclear and cytoplasmic hypertrophy. Immature microgamonts usually had folded cytoplasm and an overall spherical to elongate shape. Mean length and width  $\pm$  SEM of immature microgamonts were  $43.3 \pm 1.6$  by  $29.0 \pm 1.1$   $\mu\text{m}$ . Mature microgamonts contained hundreds of microgametes, lacked visible cytoplasmic folds, and measured  $52.8 \pm 4.7$  by  $43.0 \pm 4.2$   $\mu\text{m}$ . Macrogamonts were spherical to ovoid and had a large nucleus and prominent nucleolus. Immature macrogamonts without visible wall-forming bodies measured  $16.0 \pm 0.5$  by  $13.3 \pm 0.2$   $\mu\text{m}$ . Mature macrogamonts had 3–8- $\mu\text{m}$  eosinophilic wall-forming bodies and measured  $24.6 \pm 0.7$  by  $19.6 \pm 0.8$   $\mu\text{m}$ . Oocysts were ovoid and had a 2–3- $\mu\text{m}$ -thick eosinophilic oocyst wall. A micropyle was present in appropriately sectioned oocysts. Oocysts measured  $27.7 \pm 1.7$  by  $19.3 \pm 0.8$   $\mu\text{m}$ . The sexual stages of *E. wyomingensis* are compared to those described previously for species of *Eimeria* infecting the bovine small and large intestines.

Coccidiosis is an economically important disease of cattle (Fitzgerald, 1972, 1975). However, little is known about the life cycles of the species of bovine coccidia. Of the 13 species of *Eimeria* that infect cattle in the United States, the endogenous life cycles are known for only *E. alabamensis*, *E. auburnensis*, *E. bovis*, *E. ellipsoidalis*, and *E. zuernii* (see Ernst and Benz, 1986; Levine and Ivens, 1986). More is not known about the coccidia infecting cattle because pure species isolates are difficult to obtain, and experimental infections are difficult to produce consistently for most species (Ernst and Benz, 1980, 1986). In the present study we describe the location and structure of the sexual stages of *E. wyomingensis* in experimentally infected calves.

## MATERIALS AND METHODS

Calves were obtained and cared for as described previously (Benz and Ernst, 1979). The calves were Holstein-Friesian or Holstein-Friesian-Angus, and were 2–4 wk old at the time they were inoculated. Feces from each calf were examined at least 3 times before the calves were inoculated and only coccidia-free calves were used in this study.

Oocysts of *E. wyomingensis* were collected, cleaned, sporulated, and counted as previously described (Ernst and Benz, 1980). Each of 12 calves was orally inocu-

lated with  $10^6$  sporulated oocysts of *E. wyomingensis*. Two calves were killed at each examination period at 24-hr intervals beginning 12 days postinoculation (PI) and ending 17 days PI. Calves were killed by injection of an euthanasia fluid. The intestinal tract was immediately removed and stretched over marks at 1-m intervals. Pieces of the intestine were taken from immediately posterior to the pylorus, immediately anterior to the ileocecal junction, and at 1-m intervals in between. Tissues were also collected from the abomasum, cecum, and upper, middle, and lower colon. Tissues were fixed in Zenker's and processed by routine histological methods, sectioned at 5–7  $\mu\text{m}$ , and stained with hematoxylin and eosin. Developmental stages in tissue sections were measured with a calibrated ocular micrometer and the measurements are reported in  $\mu\text{m}$  as means  $\pm$  SEM followed by the ranges and number of stages measured (n) in parentheses. In addition to tissues for histology, several sections of intestine were collected from each calf for observation of living stages in mucosal smears using Nomarski interference-contrast microscopy. Mucosal smears were observed at room temperature.

## RESULTS

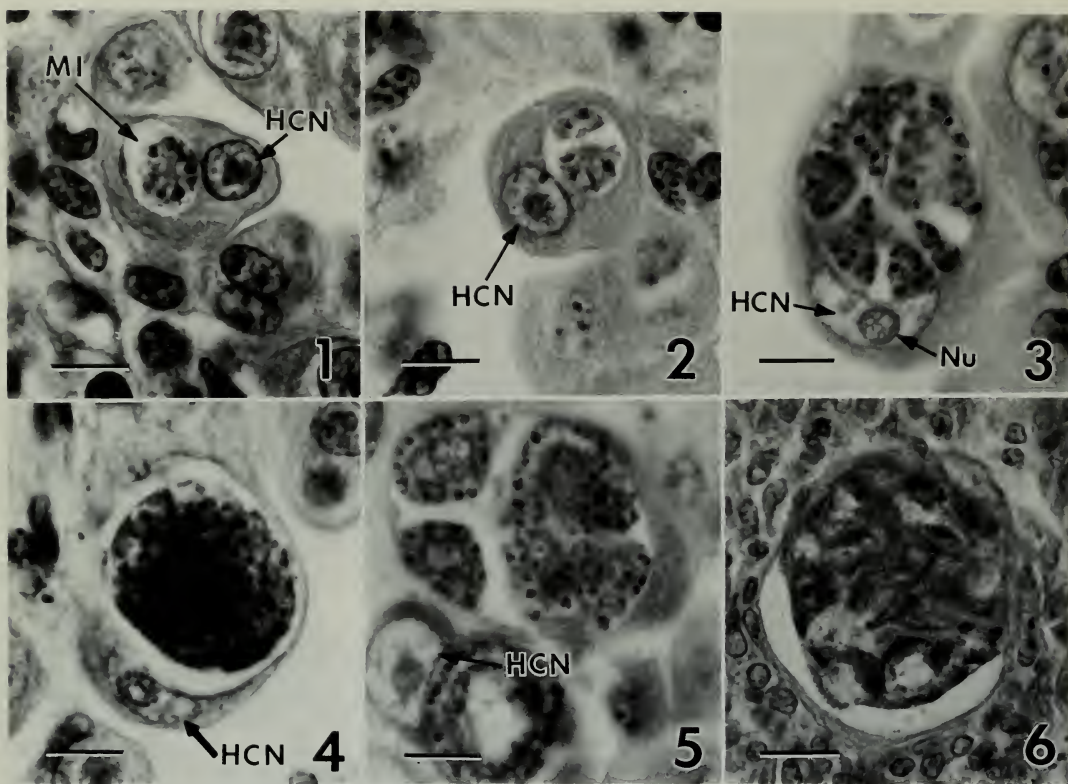
Clinical signs and gross lesions were not seen in any calves. Tissues from 7 of the 12 calves contained sexual stages of *E. wyomingensis*. No stages of *E. wyomingensis* were seen in the 5 other calves. Sexual stages ranged from immature gamonts that were present at 12 days PI to unsporulated oocysts present at 15 days PI. Sexual stages were located in host cells in the lamina propria (Figs. 1–12) of the villi in the distal 5 m of the small intestine. Developmental stages were not observed in other tissues examined. Infected

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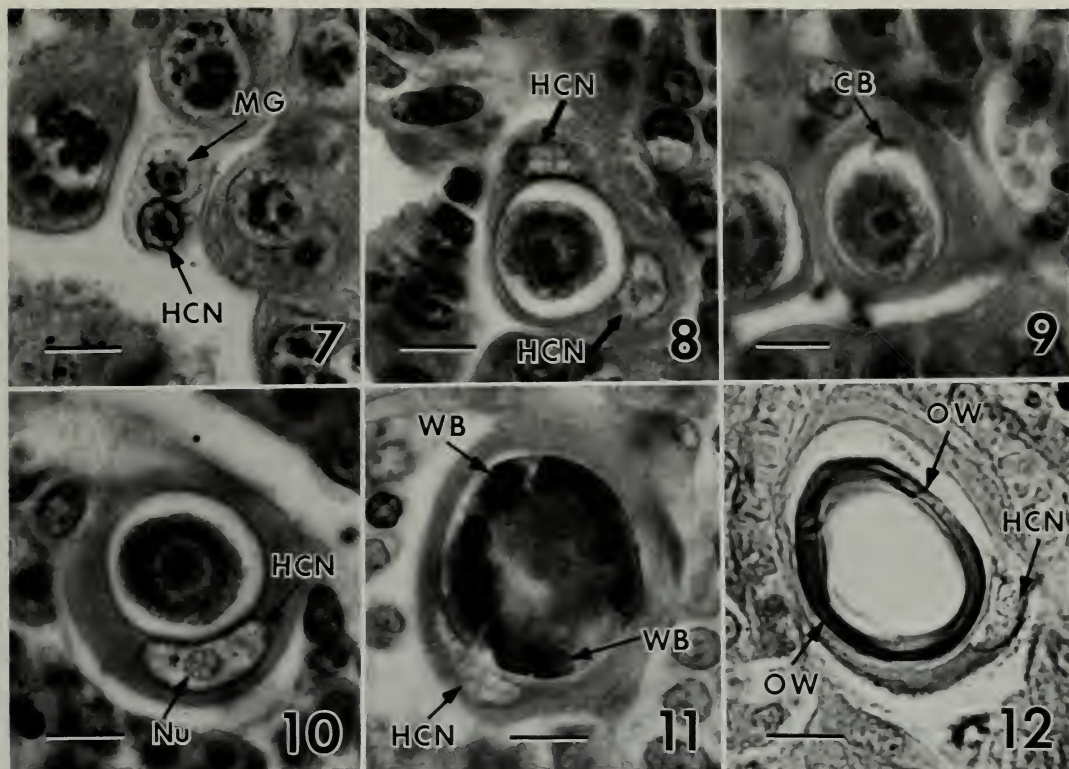
FIGURES 1-6. Microgamonts of *Eimeria wyomingensis* from experimentally infected calves. 1. Early microgamont (MI) within a host cell that has a slightly enlarged spherical nucleus (HCN). Bar = 10  $\mu$ m. 2. Early microgamont that has begun folding of the cytoplasm. Note the enlarged spherical host cell nucleus (HCN) and the rounded appearance of the infected cell. Bar = 10  $\mu$ m. 3. Maturing microgamont with several cytoplasmic folds. Note the crescent-shaped host cell nucleus (HCN) and large vesicular nucleolus (Nu). Bar = 10  $\mu$ m. 4. Maturing microgamont that has no apparent cytoplasmic folds. Note the slightly crescent-shaped host cell nucleus (HCN). Bar = 10  $\mu$ m. 5. Microgamont with cytoplasmic folds above a microgamont lacking folds. Note the enlarged spherical host cell nucleus (HCN) of the lower microgamont. Bar = 10  $\mu$ m. 6. Mature microgamont with microgametes arranged in comblike whorls. Note the lack of cytoplasmic folding. Bar = 20  $\mu$ m.

cells were usually clustered beneath the tips of the villi and in the distal  $\frac{1}{3}$  of the villi. Only in heavy infections were infected cells in portions of the villi near the crypts. A mild infiltration of lymphocytes, neutrophils, and eosinophils was associated with developmental stages of *E. wyomingensis*.

Infected host cells underwent many structural changes. The host cells and their nuclei became enlarged (Figs. 1-12). The cytoplasm of infected host cells became moderately more eosinophilic and had a finely layered appearance. Enlarged host cell nuclei were spherical (Figs. 1, 2, 5, 7) or elongate to crescent-shaped (Figs. 3, 4, 8, 10-12) and little heterochromatin was present. Rarely, more than 1 nucleus was present within a host cell (Fig. 8) (confirmed in mucosal smears). The nucleoli were also enlarged and often appeared

vesicular (Figs. 3, 4, 10). Host cell nuclei of cells containing microgamonts measured  $15.4 \pm 0.7$  by  $7.9 \pm 0.5$   $\mu$ m (10.0-20.0 by 5.0-12.0  $\mu$ m,  $n = 18$ ) and host cell nuclei of cells containing macrogamonts measured  $12.7 \pm 0.4$  by  $8.4 \pm 0.3$   $\mu$ m (9.0-17.0 by 6.0-12.0  $\mu$ m,  $n = 26$ ).

Early microgamonts were spherical and had no cytoplasmic folds (Fig. 1). The number of nuclei increased and the cytoplasm of most microgamonts became folded (Figs. 2, 3, 5) as development proceeded. These cytoplasmic folds were apparently a result of invaginations of the plasma membrane of the developing microgamont. However, many microgamonts had progressed to an advanced state of development without this folding (Fig. 4) and all fully mature microgamonts observed did not have blastophores or visible cytoplasmic folds (Fig. 6). Mi-



FIGURES 7–12. Macrogamonts and oocyst of *Eimeria wyomingensis* from experimentally infected calves. 7. Host cell with a slightly enlarged spherical nucleus (HCN) and a young macrogamont (MG). Bar = 10  $\mu$ m. 8. Maturing macrogamont within a host cell that has 2 nuclei (HCN). Bar = 10  $\mu$ m. 9. Maturing macrogamont that has a crescent body (CB) in the parasitophorous vacuole. Bar = 10  $\mu$ m. 10. Maturing macrogamont within a rounded host cell that has a crescent-shaped host cell nucleus (HCN) and vesicular nucleolus (Nu). Bar = 10  $\mu$ m. 11. Macrogamont with wall-forming bodies (WB). Note elongate host cell nucleus (HCN). Bar = 10  $\mu$ m. 12. Oocyst demonstrating a thick oocyst wall (OW) and no sporont. Note elongate host cell nucleus (HCN). Bar = 10  $\mu$ m.

crogamonts exhibiting cytoplasmic folding were spherical to elongate and measured  $40.3 \pm 1.6$  by  $29.0 \pm 1.1$   $\mu$ m (27–60 by 19–42  $\mu$ m,  $n = 30$ ). Mature microgamonts contained hundreds of microgametes that were arranged in comblike whorls on the surface of the microgamont. Mature microgamonts measured  $52.8 \pm 4.7$  by  $43.0 \pm 4.2$   $\mu$ m (42–65 by 30–52  $\mu$ m,  $n = 5$ ).

Macrogamonts were uninucleate, spherical to ovoid, and contained a large nucleus with a prominent nucleolus (Figs. 7–10). Young macrogamonts did not have visible wall-forming bodies and measured  $16.0 \pm 0.5$  by  $13.3 \pm 0.2$   $\mu$ m (13–26 by 11–15  $\mu$ m,  $n = 30$ ). Mature macrogamonts had numerous 3–8- $\mu$ m, highly eosinophilic, wall-forming bodies (Fig. 11) and measured  $24.6 \pm 0.7$  by  $19.6 \pm 0.8$   $\mu$ m (19–30 by 16–27  $\mu$ m,  $n = 25$ ). Type 1 and 2 wall-forming bodies could not be differentiated in sections or

mucosal smears. Occasionally (<3%), a crescent body could be observed in the parasitophorous vacuole of macrogamonts (Fig. 9). Crescent bodies were not observed in other developmental stages of *E. wyomingensis*.

Oocysts of *E. wyomingensis* were easily recognized because of their 2–3- $\mu$ m-thick eosinophilic oocyst wall (Fig. 12). The sporont usually was lost during tissue processing and usually was not visible in most oocysts observed in tissue sections. Oocysts usually were ovoid, and occasionally a micropyle was visible in sectioned oocysts. Oocysts measured  $27.7 \pm 1.7$  by  $19.3 \pm 0.8$   $\mu$ m (22–36 by 16–22  $\mu$ m,  $n = 7$ ).

#### DISCUSSION

Infection of calves with  $10^6$  *E. wyomingensis* oocysts did not cause disease in the present study. This is similar to the study of Ernst and Benz



(1980) in which calves were given up to  $10^7$  oocysts with no ill effects. However, Courtney et al. (1976) reported diarrhea flecked with blood in 2 calves, and diarrhea alone in 3 calves of 10 calves given  $1-2 \times 10^6$  *E. wyomingensis* oocysts. None of the calves died. The onset or duration of their clinical illness was not reported, making interpretation of the observed clinical signs difficult.

Because of the size, structure, and location of sexual stages, *E. wyomingensis* can be readily differentiated from the other species of bovine coccidia with known life cycles. *Eimeria wyomingensis* can be differentiated from *E. bovis* and *E. zuernii* of cattle because sexual stages of *E. bovis* and *E. zuernii* occur in epithelial cells of the cecum, colon, and rectum of cattle (Hammond et al., 1946, 1963a; Stockdale, 1976). *Eimeria wyomingensis* can be differentiated from *E. alabamensis* because sexual stages of *E. alabamensis* are located in the nuclei of epithelial cells usually within the lower 7 m of the small intestine (Davis et al., 1957). *Eimeria wyomingensis* can be differentiated from *E. ellipsoidalis* because sexual stages of *E. ellipsoidalis* are located in epithelial cells that line the crypts of the posterior  $\frac{2}{3}$  of the small intestine (Hammond et al., 1963b). Additionally, the mature sexual stages of *E. wyomingensis* are larger than those of *E. alabamensis*, *E. bovis*, *E. ellipsoidalis*, and *E. zuernii*.

The coccidian of cattle that *E. wyomingensis* most nearly resembles in developmental cycle is *E. auburnensis* (Hammond et al., 1961; Davis and Bowman, 1962; Chobotar and Hammond, 1969). Sexual stages of these species develop within cells in the lamina propria in the terminal small intestine. The most striking difference between the 2 species is in the size of the mature microgamonts, with those of *E. auburnensis* being much larger. Hammond et al. (1961) reported that mature microgamonts of *E. auburnensis* were 83.2–102.5 by 48.0–67.2  $\mu\text{m}$ . Davis and Bowman (1962) reported that mature microgamonts of *E. auburnensis* were 35.7–287.5 by 27.5–150.0  $\mu\text{m}$ , and some were grossly visible. Chobotar and Hammond (1969) reported that mature microgamonts of *E. auburnensis* were 42.0–109.2 by 60.9–150.6  $\mu\text{m}$ . In the present study, mature microgamonts of *E. wyomingensis* were 42.0–65.0 by 30.0–52.0  $\mu\text{m}$ . Crescent bodies were observed in both macrogamonts and microgamonts of *E. auburnensis* (Chobotar and Hammond, 1969), whereas, in our study crescent bodies were only

observed in macrogamonts and only in a minority of these stages. The function of crescent bodies is unknown. Host cells infected with *E. wyomingensis* undergo nuclear and cytoplasmic hypertrophy, whereas, those infected with *E. auburnensis* undergo nuclear hypertrophy with minimal cytoplasmic enlargement (Chobotar and Hammond, 1969). Binucleate host cells were not reported for *E. auburnensis* and the hypertrophic host cell nuclei contained prominent (condensed) nucleoli (Chobotar and Hammond, 1969), whereas, in the present study binucleate host cells were observed and the nucleoli of hypertrophic host cell nuclei were often vesicular.

Binucleate host cells have been reported for enterocytes infected with developmental stages of *E. tuskegensis* in cotton rats (Current et al., 1981). These host cell nuclei were not hypertrophic and were structurally normal. The mechanism responsible for the observed parasite-induced binucleate condition *in vivo* is not known. However, in cell cultures it has been suggested that developmental stages of *E. magna* block cytokinesis, but not karyokinesis, which results in up to 40% of the infected host cells being binucleate (Speer et al., 1973).

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## FATAL CARYOSPORA BIGENETICA (APICOMPLEXA: EIMERIIDAE) INFECTIONS IN COTTON RATS, SIGMODON HISPIDUS

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**ABSTRACT:** Four groups of cotton rats, *Sigmodon hispidus*, were shown to be suitable secondary hosts for the viperid coccidium, *Caryospora bigenetica*, following oral inoculation of a mixture of oocysts and sporocysts. Swelling of the face, ears, and scrota and hemorrhagic ears were the predominant clinical signs and some cotton rats died in 3 of 4 experiments. Developmental stages of *C. bigenetica* were found in connective tissue components of the ear, nose, cheeks, anal skin, scrotum, and penile sheath of all cotton rats in which these tissues were examined. Additionally, developmental stages of *C. bigenetica* were found in connective tissue components of the following tissues examined from some cotton rats: tongue, lung, testicle, epididymis, rectum, base of the tail, footpad, and bone marrow. The present study shows that *C. bigenetica* can be pathogenic for cotton rats and demonstrates many new anatomic sites for developmental stages of this parasite in the secondary host.

Several studies have demonstrated that members of the coccidian genus *Caryospora* (Apicomplexa: Eimeriorina) have facultative heteroxenous life cycles (Stockdale and Cawthorn, 1981; Cawthorn and Stockdale, 1982; Wacha and Christiansen, 1982; Upton et al., 1984; Upton and Barnard, 1986, 1988). In mice (secondary hosts) experimentally infected with oocysts of either *C. simplex* or *C. bigenetica* from snakes (primary hosts), asexual and sexual multiplication followed by sporogony and excystation occur in connective tissues of the nose, cheeks, tongue, and scrota (Wacha and Christiansen, 1982; Upton et al., 1984; Upton and Barnard, 1988). Sporozoites that excyst from *in vivo*-produced oocysts enter connective tissue or macrophage-like host cells that are induced to transform into spherical to broadly ellipsoidal cells called caryocysts (Wacha and Christiansen, 1982; Upton et al., 1984, 1985; Upton and Barnard, 1988). Caryocysts may contain 1 or several sporozoites. Caryocysts present in the tissues of experimentally infected mice are infective for snakes (Wacha and Christiansen, 1982; Upton and Barnard, 1986) and for mice (Upton et al., 1985).

Clinical signs in mice infected with *C. bigenetica* consist of swelling of the face, tongue, and scrotum (Wacha and Christiansen, 1982; Upton and Barnard, 1988). Wacha and Christiansen (1982) observed deaths in 2 of 5 male mice 10 days postinoculation of 6,000 *C. bigenetica* oocysts, whereas Upton and Barnard (1988) found no deaths in 27 male mice given 300,000 or 10 male mice given 200,000 *C. bigenetica* oocysts. Neither of these studies examined the responses of female mice to inoculation of *C. bigenetica* oocysts.

Because a previous study indicated cotton rats were susceptible to *C. simplex* infection (Upton et al., 1984), we decided to determine the extent to which both male and female cotton rats (*Sigmodon hispidus*) were susceptible to experimental infection with *C. bigenetica*.

### MATERIALS AND METHODS

Oocysts of *C. bigenetica* were obtained from the feces of a naturally infected northern copperhead, *Agkistrodon contortrix mokasen*. Methods used to sporulate and initially concentrate oocysts for inoculations were identical to those described by Upton and Barnard (1988). Oocysts were further cleaned by flotation in Sheather's sugar solution (spec. gravity 1.18) and stored in 2.5% (w/v) potassium dichromate solution for 9 mo at 4 C before use. Prior to inoculation of cotton rats, the potassium dichromate solution was removed by centrifugation and the oocysts incubated in 5.25% (v/v) sodium hypochlorite solution for 10 min. The sodium hypochlorite was removed by centrifugation in Hanks' balanced salt solution (HBSS) (GIBCO Laboratories, Grand Island, New York) and the oocyst walls were broken by grinding in a motor-driven Teflon-coated tissue grinder. The number of oocysts and sporocysts present in the inoculum was determined using a hemacytometer. A mixture of oocysts and sporocysts was used to infect our cotton rats because Upton et al.

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(1984) demonstrated that more numerous developmental stages of *C. simplex* were present in mice if they were inoculated with a mixture of oocysts and sporocysts than with oocysts alone.

The susceptibility of cotton rats to *C. bigenetica* infection was examined in 4 groups of experimentally infected cotton rats. Group 1 contained 4 males and 4 females that were each inoculated with 800,000 oocysts and 800,000 sporocysts. Group 2 contained 6 males and 6 females that were each inoculated with 400,000 oocysts and 300,000 sporocysts. Group 3 contained 4 males that were each inoculated with 300,000 oocysts and 100,000 sporocysts. Group 4 contained 5 males and 5 females that were each inoculated with 150,000 oocysts and 50,000 sporocysts. A control group (group 5) contained 1 male and 1 female that were not inoculated with *C. bigenetica*. Cotton rats in groups 1–3 and 5 were 7–8 wk old when inoculated and cotton rats in group 4 were 5–6 wk old when inoculated. Cotton rats were housed in plastic cages with wood shavings for bedding. Water and commercial rat chow were provided *ad libitum*.

The days postinoculation (DPI) when cotton rats in inoculated groups were examined at necropsy are presented in Table I. Control cotton rats were examined at necropsy 2 wk after they were chosen to be the controls. Tissues collected at necropsy from all cotton rats were: ears, nose, cheeks, and tongue. The vulva region was collected from females and the scrotum (usually with epididymis present), and penile sheath were collected from males. The following tissues were also collected from cotton rats in group 1 and group 5: brain, heart, lung, liver, spleen, kidney, pancreas, and gastrocnemius. The testicles were collected from males in groups 1, 2, and 5. Other tissues that were collected from a minority of cotton rats in all groups were: rectum, anal skin, base of the tail, and footpad. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 8  $\mu$ m, and stained with hematoxylin and eosin for microscopic examination. Tissues containing bone were decalcified in 10% (w/v) ethylenediaminetetraacetic acid in deionized water (final pH 7.2) for 14–21 days before processing.

## RESULTS

The clinical response to infection was similar in all 4 groups of inoculated cotton rats. Controls remained healthy throughout the study. The facial tissues, ears, scrota, and footpads were swollen 8 DPI. Facial swelling and footpad swelling were more pronounced in males than females. Cotton rats became less active and often burrowed under their bedding. Hemorrhagic lesions were first observed on the ears 11 DPI and were present in both male and female cotton rats. These lesions then scabbed. Cauliflower-like ears developed in some cotton rats examined after 20 DPI and gross swellings of tissues had resolved at this time.

Deaths or comatose cotton rats were present in groups 1, 2, and 4 (Table I). Comatose females

were not observed in any group. With the exception of 1 male cotton rat that was found dead 6 DPI, examination of all other males that were found dead or were comatose (11 of 19, 57.9%) (Table I) indicated that infection due to *C. bigenetica* was the cause of their conditions. Examination of tissues from 1 of the 2 females that was found dead (10 DPI, group 1) (Table I) indicated that *C. bigenetica* infection was probably not the cause of death. The cause of death of these 2 female cotton rats was not determined. Two males (group 1) died while under observation 10 DPI before they could be euthanized. These males and others that were euthanized when comatose exhibited similar clinical signs prior to becoming comatose. These males had markedly swollen faces, ears, and scrota. One or both eyes were often encrusted shut and the abdomen was sunken. They moved with a staggering gait when probed with forceps. These cotton rats felt cold when held for examination. Breathing was labored and the cotton rats were usually in lateral recumbency prior to death.

Developmental stages were first seen in cotton rats examined 10 DPI and consisted mainly of Type III merozoites (Upton and Barnard, 1988) with a few young sexual stages. Sporulated oocysts and caryocysts were first seen 12 and 13 DPI, respectively. Caryocysts were the predominant stage observed in cotton rats examined after 16 DPI; however, stages resembling degenerated oocysts were observed in a male cotton rat 22 DPI. Only caryocysts were observed in female cotton rats examined 23, 29, and 34 DPI.

Developmental stages of *C. bigenetica* were observed in macrophage-like host cells in connective tissue components of the ear, nose, cheeks, scrotum, and penile sheath in all infected cotton rats examined histologically ( $n = 31$ ). Developmental stages were also observed in the lungs (3 of 8) (Fig. 1), testicle (7 of 8), epididymis (13 of 15), anal skin (9 of 9), submucosa and lamina and tunica propria of the rectum (7 of 9) (Fig. 2), base of the tail (14 of 22), footpad (16 of 22), tongue (2 of 26), and bone marrow of the tarsal bones of the foot (7 of 9). Developmental stages of *C. bigenetica* were not observed in control cotton rats or in any other tissues from experimentally infected cotton rats.

## DISCUSSION

The present study shows that cotton rats are suitable secondary hosts for *C. bigenetica* and that the appearance and time of occurrence of



TABLE I. Sex and status of 4 groups of cotton rats inoculated with oocysts and sporocysts of *Caryospora bigenetica* and examined at necropsy on various days postinoculation (DPI).

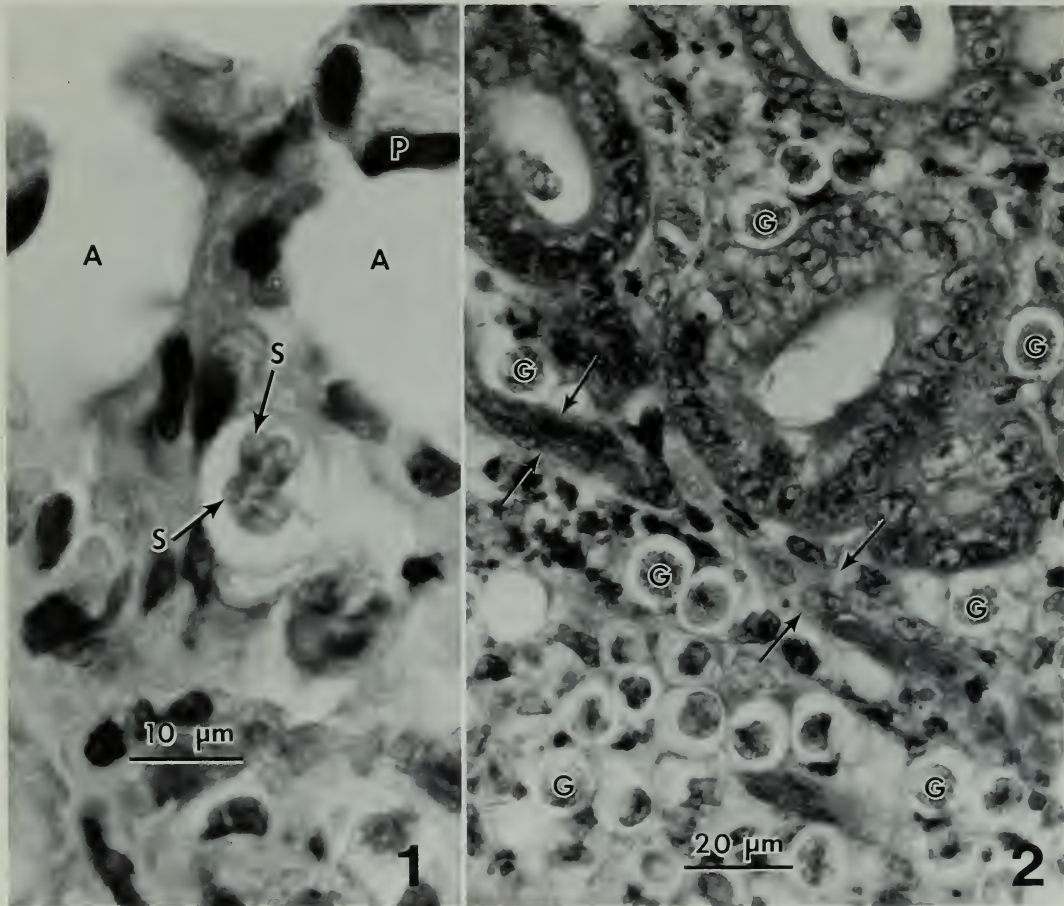
Group 1*			Group 2			Group 3			Group 4		
DPI	Sex†	Status‡	DPI	Sex	Status	DPI	Sex	Status	DPI	Sex	Status
10	M	D	11	M	FD§	10	M	E	6	M	FD§
10	M	D	11	F	FD§	12	M	E	11	M	EC
10	F	FD	12	M	E	14	M	E	13	M	EC
11	M	FD	12	F	E	16	M	E	13	M	EC
12	M	EC	13	M	FD				14	F	E
16	F	E	13	M	EC				15	F	E
16	F	E	13	M	EC				22	M	E
34	F	E	14	M	E				23	F	E
			14	F	E				23	F	E
			16	F	E				23	F	E
			16	F	E						
			23	F	E						

\* Group 1 received 800,000 oocysts and 800,000 sporocysts, group 2 received 400,000 oocysts and 300,000 sporocysts, group 3 received 300,000 oocysts and 100,000 sporocysts, and group 4 received 150,000 oocysts and 50,000 sporocysts.

† M = male, F = female.

‡ D = died while under observation, E = euthanized, EC = euthanized while comatose, FD = found dead.

§ No tissues collected for histopathology.



FIGURES 1, 2. Developmental stages of *Caryospora bigenetica* in experimentally infected cotton rats. Hematoxylin and eosin-stained tissue sections. 1. Sporulated oocyst in the interstitium of the lung. Note sporozoites (S), alveoli (A), and a pneumocyte (P), 16 DPI. 2. Section of the rectum demonstrating numerous gamonts (G) in the submucosa and tunica propria (arrows point to muscularis mucosae), 13 DPI.

developmental stages is similar to that reported for experimentally infected mice (Wacha and Christiansen, 1982; Upton and Barnard, 1988). Clinical signs of infection in cotton rats are also similar to those reported in mice except that ear lesions have not been reported in mice. We observed a high mortality rate (57.9%) in male cotton rats attributable to *C. bigenetica* infection in the present study. Wacha and Christiansen (1982) found that 40% (2 of 5) of experimentally infected male mice died due to *C. bigenetica* infection. However, Upton and Barnard (1988) did not observe mortality in 10 male mice experimentally infected with oocysts of the same isolate of *C. bigenetica* used in the present study or 27 male mice inoculated with *C. bigenetica* oocysts from a diamondback rattlesnake, *Crotalus adamatus*. We have not produced mortality in male mice by inoculation of up to 300,000 oocysts of the isolate of *C. bigenetica* used in the present study or up to 300,000 oocysts of *C. bigenetica* isolated from a naturally infected timber rattlesnake (*Crotalus horridus*) (C. A. Sundermann, unpubl. data). The reasons for the lack of agreement in results of experimental infections in male mice may be due to differences in the pathogenicity of isolates of *C. bigenetica* used or may be an artifact of the low number of male mice examined by Wacha and Christiansen (1982). Results of our study suggest that *C. bigenetica* infection is more pathogenic for male cotton rats than male mice.

Our study shows that female cotton rats develop less severe clinical signs of infection and are less likely to die from infections than are males. This suggests a sex-related resistance to infection with *C. bigenetica*, however, more cotton rats would have to be examined in order to draw definitive conclusions.

Previous studies had failed to demonstrate developmental stages of *C. bigenetica* (Wacha and Christiansen, 1982; Upton and Barnard, 1988) or *C. simplex* (Upton et al., 1984) in visceral, reproductive, hemopoietic, or intestinal tissues.

Our findings indicate that these tissues do become infected but usually contain fewer parasites than other tissues such as the nose, cheeks, or scrotum. One reason previous studies have failed to find developmental stages in these tissues may have been because of lower inoculating doses and because many of the tissues we examined were not examined by other researchers.

#### ACKNOWLEDGMENT

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## ECTOPARASITES OF SYMPATRIC COTTONTAILS (*SYLVILAGUS AUDUBONII* NELSON) AND JACK RABBITS (*LEPUS CALIFORNICUS* MEARNES) FROM THE HIGH PLAINS OF EASTERN NEW MEXICO

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**ABSTRACT:** Eight species of ectoparasites were recovered from 35 *Sylvilagus audubonii* and 35 *Lepus californicus* occurring sympatrically near the Clovis–Portales area of eastern New Mexico. Recovered were Anoplura (*Haemodipsus setoni*), Diptera (*Cuterebra lepusculi* and *Cuterebra ruficus*), Siphonaptera (*Echidnophaga gallinacea* and *Euhoplosyllus glacialis*), and Acari (*Ornithodoros parkeri*, *Dermacentor parumapertus*, and *Haemaphysalis leporispalustris*). Jaccard's index showed a 50% ectoparasitic overlap with *H. setoni*, *E. glacialis*, *D. parumapertus*, and *H. leporispalustris* present on both host species. *Cuterebra lepusculi*, *E. gallinacea*, and *Ornithodoros parkeri* were taken only from *S. audubonii*, whereas *C. ruficus* occurred only on jack rabbits. *Euhoplosyllus glacialis* was the only species to demonstrate a preference for sex of host, occurring more abundantly on females.

Because plague is a problem in New Mexico, most studies on the ectoparasites of *Lepus californicus* and/or *Sylvilagus audubonii* in New Mexico have involved only fleas (Kartman, 1960; Forcum et al., 1969; Graves et al., 1978). Prevalence and intensity of ectoparasites from both hosts have been reported for northern (Morlan, 1955) and southwestern (Rodriguez, 1977) New Mexico. The objectives of this study are (1) to describe ectoparasites from sympatric cottontails and jack rabbits from the high plains of eastern New Mexico, and (2) to show existence of host preference, host sex preference, and ectoparasite overlap where it may exist.

Thirty-five *Sylvilagus audubonii* and 35 *Lepus californicus* were examined for ectoparasites. All hosts were collected within 18.6 km of Clovis, Curry Co., New Mexico, during May through November of 1985. Host habitat was a high, rolling, short-grass prairie dominated by blue grama (*Bouteloua gracilis*), side-oats grama (*Bouteloua curtipendula*), purple three-awn (*Aristida purpurea*), sand dropseed (*Sporobolus cryptandrus*), and kochia weed (*Kochia scoparia*), with the predominant shrubs being sand sage (*Artemisia filifolia*) and honey mesquite (*Prosopis glandulosa*).

Collections were made using a 22-caliber rifle with solid tip ammunition. Immediately after shooting, all hosts were placed individually in sealed and labeled plastic garbage bags and frozen. A regimen of visual inspection of bag and host, brushing, then skinning, and finally dissection

of the hide in 20% KOH for 24 hr was used to recover all ectoparasites. Ectoparasites were treated in the usual manner and mounted in Euparal, and cuterebrid larvae were stored in 70% ethanol.

Skeletons of *S. audubonii* and *L. californicus* were deposited in the Natural History Museum, Eastern New Mexico University (Nos. 9694–9700). Parasites were accessioned in the Eastern New Mexico University medical zoology collection (Nos. 1708–1842) and the U.S. National Parasite Collection (Beltsville, Maryland; Nos. 79832–79839).

Fleas and cuterebrid larvae were identified by Dr. Robert E. Lewis and Dr. Craig Baird, respectively. Use of the terms prevalence, mean intensity, and intensity follow Margolis et al. (1982). Chi-square, *F*-distribution, and Jaccard's index (Leong and Holmes, 1981) of parasitic overlap were used to analyze host–parasite interactions.

Seventy-four percent of 969 ectoparasites were recovered from *S. audubonii* and 26% from *L. californicus*. Included among these were Anoplura (1 species), Diptera (2), Siphonaptera (2), and Acari (3) (Table I).

### Anoplura

*Haemodipsus setoni* was recovered from both host species, but 78% of 123 specimens were on *L. californicus*. Four jack rabbits harbored 17–27 lice, and no more than 1–5 lice were recovered from the remaining hares. Overdispersion was even greater on *S. audubonii* where 25 of 27 lice were taken from a single host. Prevalence of in-

TABLE 1. *Ectoparasites of sympatric Sylvilagus audubonii and Lepus californicus in eastern New Mexico.*

Ectoparasite	<i>S. audubonii</i>		<i>L. californicus</i>	
	Prevalence	Intensity	Prevalence	Intensity
Anoplura				
<i>Haemodipsus setoni</i>	8.6 (35)*	9.0 (1-25)†	23 (35)*	12.0 (1-27)†
Diptera				
<i>Cuterebra lepusculi</i>	14.3	2.4 (1-4)	—	—
<i>Cuterebra ruficrus</i>	—	—	22.9	1.1 (1-2)
Siphonaptera				
<i>Echidnophaga gallinacea</i>	5.7	5.0 (1-9)	—	—
<i>Euhoplosyllus glacialis</i>	88.6	17.5 (1-93)	31.4	6.9 (1-22)
Acarina				
<i>Dermacentor parumapertus</i>	8.6	1.3 (1-2)	28.6	7.2 (1-14)
<i>Haemaphysalis leporispalustris</i>	62.9	5.7 (1-27)	20.0	1.1 (1-2)
<i>Ornithodoros parkeri</i>	2.9	1.0	—	—

\* Percent hosts infected with total number of hosts examined in parentheses.

† Mean number and range in number of parasites per infected host.

festation occurred independent of host species ( $\chi^2 = 2.69$ ;  $P > 0.10$ ). Comparisons of mean intensities between *L. californicus* and *S. audubonii* could not be accurately analyzed due to small sample sizes. Four of the 8 jack rabbits infested with *H. setoni* harbored only this ectoparasite.

Immature *H. setoni* represented 48% (13/27) of the lice recovered from cottontails and 59% (57/96) from jack rabbits. Comparisons of mean intensities of *H. setoni* among female (16) and male (9) jack rabbits confirmed that presence of *H. setoni* on *L. californicus* was independent of host sex ( $F = 1.5$ ;  $CV = 5.41$ ).

#### Diptera

Cuterebids were the least common ectoparasites. Twelve *C. lepusculi* larvae were recovered from *S. audubonii* and 9 *C. ruficrus* larvae were found in *L. californicus* (Table I). In decreasing order of occurrence, *C. ruficrus* larvae were recovered from subcutaneous tissues in the dorsal thoracic (4), right lumbar (3), right lateral scapular (1), and ventral cervical (1) regions of the body. In *S. audubonii*, *C. lepusculi* larvae were found in the dorsal thoracic (6), ventral abdominal wall (2), left ventral thoracic wall (1), right lateral scapular (1), and perineal areas (1).

#### Siphonaptera

Fleas were the most common ectoparasites (620/969; 64%). Although few in number, all *E. gallinacea* were recovered from cottontails (Table I). Prevalence of infestation by *E. glacialis* was much higher among *S. audubonii* than *L.*

*californicus* (Table I). Prevalence ( $\chi^2 = 23.81$ ;  $P < 0.0005$ ) as well as mean intensities of infestation ( $F = 34.75$ ;  $CV = 2.70$ ) were host-species related.

More female than male *E. glacialis* were recovered from *S. audubonii* (62%) than from *L. californicus* (66%). Mean intensities of *E. glacialis* on female cottontails ( $\bar{x} = 21.8$ ) were greater than on male ( $\bar{x} = 12.4$ ) cottontails ( $F = 4.31$ ;  $CV = 2.53$ ). Three of the hosts were gravid and in advanced stages of pregnancy and demonstrated overdispersion among *E. glacialis*. There were no *E. glacialis* on 1 pregnant female, 19 on another, and 93 *E. glacialis* on the other. Of these overdispersed fleas, 64% were females from each of the respective hosts.

Female *E. glacialis* were more common (68% of 338) on female than on male *S. audubonii*. The mean intensity of female *E. glacialis* on female cottontails ( $\bar{x} = 15.3$ ) was greater than on male ( $\bar{x} = 7.9$ ) cottontails ( $F = 4.2$ ;  $CV = 2.55$ ). Similarly, more male *E. glacialis* (69% of 206) were found on female than on male cottontails. The mean intensities of male *E. glacialis* on female cottontails (9.5) was greater than on male (5.7) cottontails ( $F = 4.91$ ;  $CV = 2.87$ ).

#### Acar

Jack rabbits were the preferred hosts of *D. parumapertus*. Ten jack rabbits were infested with 95% of the *D. parumapertus*. Prevalence ( $\chi^2 = 4.63$ ;  $P < 0.05$ ) and mean intensity ( $F = 413$ ;  $CV = 8.79$ ) of *D. parumapertus* was host-species related. Additionally, 75% (57/76) of the *D. parumapertus* recovered from jack rabbits were males



and 5 (19; 26.3%) of the females were blood engorged and were taken from the ears of jack rabbits. Finally, 3 of 10 jack rabbits were infested with only this ectoparasite species.

*Haemaphysalis leporispalustris* was the predominant tick on *S. audubonii* (94% of 134). Variation in ranges of infestation were equally significant with the range on *S. audubonii* being much greater than on *L. californicus* (Table I). Prevalence ( $\chi^2 = 13.25$ ;  $P < 0.0005$ ) and mean intensity ( $F = 1,081.25$ ;  $CV = 3.87$ ) of infestation were significantly greater on cottontails.

Of the *H. leporispalustris* recovered from cottontails, 18 (116; 15.5%) were adult females, 43 (37.1%) were adult males, and 55 (47.4%) were nymphs. Nymphs were insignificantly distributed between host sexes.

Jaccard's index of similarity showed a 50% overlap among the 8 ectoparasite species. Aside from the apparent accidental association between *O. parkeri* and *S. audubonii*, *C. lepusculi* and *E. gallinacea* demonstrated monoxenic host preference toward cottontails, and a similar relationship existed between *C. ruficrus* and *L. californicus*.

Twelve species of fleas, totaling 2,906 in number, were collected from cottontails in neighboring Chavez county (Forcum et al., 1969). As in the present study, *E. glacialis* was the most abundant species. Low incidence levels of *E. gallinacea* observed by Forcum et al. (1969) are similar to our observations. Pfaffenberger and Wilson (1985) did recover *E. glacialis* and *E. gallinacea* from 11 of 14 (78%) and 1 of 14 (7%) cottontails, respectively. Our results concur with their observations.

The present study provides the first report of *H. setoni* from cottontails and jack rabbits in New Mexico.

The fly *C. lepusculi* has been recorded from its type host (*S. audubonii*) in New Mexico (Sabrosky, 1986). Rodriguez (1977) recovered 5 cuterebrids from 3 cottontails in Grant County compared to 12 larvae from 5 hosts in our study. Although he did not specifically identify them because of demonstrated host preference (Sabrosky, 1986), they too were probably *C. lepusculi*. Jack rabbits are recorded hosts to immatures of *Cuterebra mirabilis* in New Mexico. Sabrosky (1986) presents collection records for this species in Albuquerque and Ft. Sumner. The latter site is approximately 43 km to the west-northwest of the present study site. Although

adults of *C. ruficrus* have been recorded from this area (Sabrosky, 1986), to our knowledge our study was the first to report the presence of its larvae from *L. californicus*.

\* Because Rodriguez (1977) did not report intensities of flea infestations, site comparisons were not indicated. Rodriguez (1977) recovered 13 *E. glacialis* from 6 hares, but he also recovered 5 *E. gallinacea* from the same number of hosts. The mean intensity of infestation by *E. glacialis* in the latter study (2.2) is lower than that observed in this study (Table I). No *E. gallinacea* were recovered from *L. californicus* in our study. *Echidnophaga gallinacea* was also recovered from jack rabbits in Santa Fe county (Morlan, 1955).

Cottontails were hosts of a greater diversity of pulicids than were jack rabbits. This difference was more than likely due to divergence in behavior. Hares do not dig or utilize burrows, but instead hide in vegetation (Walker, 1968). Moreover, their litters (2–3) are hidden among dense vegetation (Walker, 1968). In comparison, cottontails rest in burrows during the day. They also rear 3–5 litters per year in burrows (Walker, 1968) where humidity levels remain statically high. A high, stable humidity is known to be required by fleas in order to survive, which probably explains why cottontails are apt to be more heavily infested with a greater diversity and density of fleas than are jack rabbits.

The fact that male cottontails harbored fewer fleas could possibly be explained by behavioral as well as physiological differences between sexes. Philandering males are less apt to be in burrows than are their female counterparts. Females, on the other hand, secrete a variety of potentially stimulating hormones (Marshall, 1981), some of which are known to effectively regulate flea behavior (Rothschild, 1965) and most of which do not occur in male cottontails.

Fewer species of fleas were observed in this study than in studies in Santa Fe (Morlan, 1955) and Grant (Rodriguez, 1977) counties. Twenty-eight cottontails from Grant County were infested with 4 species of fleas (*C. inaequalis*, *E. glacialis*, *E. gallinacea*, and *Meringis dipodomys*), 2 of which were also observed in our study (Table I). However, 7 species of fleas were recorded from *S. audubonii* by Morlan (1955). He observed a broad range in percent prevalence (0.01–79%) and mean intensity (1.0–23.5) of infestation by the different species of fleas among 170 cottontails. According to Morlan (1955), *E.*

*glacialis* was the most prevalent species of flea and appeared with greatest mean intensity. The only species common to all 3 studies was *E. glacialis*. It appeared with a higher percent prevalence (88.6) but a lower mean intensity (17.6) in this study than in Santa Fe County. No comparative data were provided by Rodriguez (1977).

We are not certain why these differences exist but feel that perhaps the lower relative humidity, higher ambient temperatures, and porous, sandy soils of eastern New Mexico are less apt to maintain humidity levels required perhaps by other species of fleas. Alternate methods of collecting hosts, duration of collection period (Morlan, 1955), host population density (Rodriguez, 1977), as well as other habitat differences could also account for the diversity in flea species and differing densities among the 3 New Mexico studies.

*Dermacentor parumapertus* has been recovered from jack rabbits in New Mexico on 2 previous occasions (Samson, 1968; Rodriguez, 1977). The 54% prevalence observed by Rodriguez (1977) was higher than in our study (Table I). Rodriguez (1977) did not recover *D. parumapertus* from 28 cottontails compared to our 9% prevalence.

*Haemaphysalis leporispalustris* was the most abundant tick species on cottontails in either study. It was more prevalent in the present study (63%) than described by Rodriguez (1977, 21%). Two of 24 (7%) jack rabbits were infested as compared to 7 of 35 (20%) hosts in this study. Rodriguez (1977) also observed the presence of 3 *Otobius megnini* on 2 (28, 7%) cottontails, which, like *O. parkeri* in the present study, are host records in this state and both might be considered accidental associations.

Finally, Rodriguez (1977) was the only one to report the occurrence of all ectoparasites from both rabbits and hares in a New Mexico study. Therefore, his study is the only one, other than our own, in which parasitic overlap can be examined. The 50% parasitic overlap observed in our eastern New Mexico study was higher than the 37.5% overlap, which occurred in southwestern New Mexico (Rodriguez, 1977). This difference could possibly be associated with the monotypic habitat type of the rolling, high plains of eastern New Mexico as compared to the variable habitat of the montane southwestern part of the state. It could also reflect variations in time and season. We have no way of knowing what impact season may have had because Rodriguez

(1977) failed to report the months during which his study was conducted.

The presence of certain ectoparasites on only 1 host may be explained on the basis of a multitude of physiological differences (Traub, 1985) between hosts. The significance of the 50% overlap may have been related to habitat preference and/or biology of the parasite. The entire life cycle of *H. setoni* occurs on the surface of its host. This anopluran, typically an ectoparasite of jack rabbits (Kim et al., 1986), may have infested cottontails as a result of the habits of both hosts to seek cover in dense vegetation (Walker, 1968). Because only 3 cottontails were infested with this louse species and where 2 of the cottontails harbored 1 louse each, it seems reasonable that while in the vegetation some of the lice may have become dislodged from jack rabbits and were subsequently acquired by cottontails. The cottontail harboring 25 lice may have been one that returned to a jack rabbit lair repeatedly. All life stages of *E. glacialis* are also spent on the surface of its host (Traub, 1985). Therefore, explanations for the presence of *H. setoni* on both hosts might apply here as well.

Although *D. parumapertus* is known to favor *L. californicus*, its presence on cottontails in our study was apparently due to host preference (Furman and Loomis, 1984). The immatures reside in vegetation and near or just within burrow entrances where they are most apt to encounter the necessary rodents, cottontails, and even an occasional jack rabbit (Furman and Loomis, 1984) to achieve sexual maturity. According to Krantz (1978) *H. leporispalustris* is a rabbit tick whose presence on its host is regulated scotopically. They drop off at night to locate other cottontails and occasional jack rabbits.

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# PHYLOGENETIC RELATIONSHIPS OF THE APICOMPLEXAN PROTIST *SARCOCYSTIS* AS DETERMINED BY SMALL SUBUNIT RIBOSOMAL RNA COMPARISON

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**ABSTRACT:** Reverse transcription of total cellular RNA was used to obtain the partial nucleotide sequence of the small subunit ribosomal RNA (srRNA) of *Sarcocystis gigantea*. The sequence was compared with the homologous sequences of 24 other eukaryotes. Phylogenetic analysis of the semiconserved regions by 4 different tree-building methods using bacteria as an outgroup all concur in showing monophyly of *Sarcocystis gigantea* and *Toxoplasma gondii* to the exclusion of all other taxa for which homologous sequences are available.

The phylum Apicomplexa contains many organisms of importance to human and veterinary medicine (Levine, 1970; Levine et al., 1980). Even though 3 genera (*Plasmodium*, *Toxoplasma*, *Sarcocystis*) in this phylum are common parasites of man and domestic animals, their evolutionary relationships to each other and to other protozoa are still subject to debate (Fayer, 1981; Baker, 1987; Editorial, 1987; Levine, 1987).

We recently used a rapid nucleotide sequencing technique to obtain the sequence of the semi-conserved regions of the small subunit ribosomal RNA (srRNA) of *Toxoplasma gondii*. A phylogenetic analysis of this sequence along with those of *Plasmodium berghei* and a range of other eukaryotes failed to reveal a close phylogenetic relationship between *T. gondii* and *P. berghei* (Johnson et al., 1987). This result therefore calls into question the monophyly of other members of the Apicomplexa.

In the present study, we have used the same technique to sequence the srRNA of *Sarcocystis gigantea* in order to determine its phylogenetic affinities. Three competing hypotheses were that *S. gigantea* is closely related to *T. gondii*, to *P. berghei*, or not to either.

## MATERIALS AND METHODS

### RNA extraction

Bulk cellular RNA was extracted and purified from sarcocysts removed from the exterior of sheep esophagi, using a modification of the method of Brooker et al. (1980) as described previously (Johnson et al., 1986). The RNA was used at a final concentration of 2.0 mg/ml.

### Oligonucleotide primers

Three rRNA primers were obtained commercially (Boehringer Mannheim GmbH-Biochemical, Mannheim, F.R.G.) and used at a final concentration of 0.1 mg/ml. The nucleotide sequences of each of the rRNA primers are: primer A (5'-GWATTACCGCG-GCKGCTG-3'), primer B (5'-CCGTCAATTCMTT-TRAGTTT-3'), and primer C (5'-ACGGGCGGT-GTGTRC-3'). Their complementary binding sites in *Escherichia coli* using the srRNA numbering system of Brosius et al. (1981) are: primer A (positions 519-536), primer B (positions 907-926), and primer C (positions 1,392-1,406).

### Dideoxynucleotide sequencing

The method used was essentially that of Lane et al. (1985). Thirty-five  $\mu$ Ci of [ $\alpha$ -<sup>35</sup>S]dATP (Amersham International plc, Buckinghamshire, U.K.) and 6.2 units of avian myeloblastosis virus reverse transcriptase (Promega Biotec Pty. Ltd., Wisconsin) were used per reaction. Reactions were heated to 90 C for 2 min prior to loading onto a 6% polyacrylamide 8 M urea 0.4-mm-thick nongradient gel. After electrophoresis at 1,200 V, gels were fixed in 10% acetic acid, then 20% methanol, and baked onto the glass plate at 110 C for 1 hr. The dried gels were then autoradiographed using Kodak X-OMAT X-ray film. At least 2 sequencing reactions and determinations were carried out for each primer to ensure the accuracy of the nucleotide sequences obtained. Two reactions were electrophoresed for 3 hr and 2 reactions were electrophoresed for 6 hr to extend the sequence obtained.

### Phylogenetic analysis

The *Sarcocystis* sequence was aligned by eye with published homologous sequences for vertebrates (*Homo*, *Mus*, *Rattus*, *Oryctolagus*, *Xenopus*), an invertebrate (*Artemia*), fungi (*Saccharomyces*, *Neurospora*), apicomplexans (*Toxoplasma*, *Plasmodium*), ciliates (*Oxytricha*, *Stylonychia*, *Tetrahymena*, *Paramecium*, *Euplotes*), a cellular slime mould (*Dictyostelium*), plants (*Oryza*, *Zea*, *Glycine*), 2 flagellates (*Trypanosoma*, *Euglena*), a dinoflagellate (*Prorocentrum*), an amoeba (*Acanthamoeba*), and a microsporidium (*Vairimorpha*) [see Johnson et al. (1987) for sources; also Vossbrinck et al. (1987) for *Vairimorpha*, Herzog and Marteau (1986) for *Prorocentrum*, and Gunderson and

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Sogin (1986) for *Acanthamoeba*]. To reduce the computer time needed for the analysis we used only 1 taxon to represent groups that have consistently formed monophyletic assemblages in previous analyses. Thus, *Homo* represented the vertebrates, *Saccharomyces* the fungi, and *Tetrahymena* the ciliates, leaving a total of 14 sequences. Homologous sequences from representatives of the Eubacteria (*Escherichia*) and of the Archaeobacteria (*Halobacterium*) from Huysmans and De Wachter (1985) formed the outgroup to root the trees. The data base is included as an Appendix. To reduce noise in the data, only the semiconserved regions in the primary structure were chosen for analysis and only regions with less than 6 continuous nucleotide deletions or insertions per block were selected (Elwood et al., 1985). A total of 369 sites was used.

Most published phylogenetic analyses of 18S rRNA sequences use a distance matrix method (e.g., Elwood et al., 1985; Lane et al., 1985; Herzog and Maroteaux, 1986; Vossbrinck et al., 1987). Although distance methods have some justification (Felsenstein, 1984), it seems that character state data are more likely to reveal the true tree topology (e.g., Penny, 1982; Sourdis and Nei, 1988). We have therefore analysed the semiconserved sequence data for these 16 taxa by both methods. The character-state data were analysed using 2 criteria—parsimony and maximum likelihood. Parsimony aims to find the tree with the minimum amount of homoplasy, whereas maximum likelihood aims to find the tree for which the data have the highest probability (Felsenstein, 1981). Two approaches were used to find the most parsimonious tree—the DNAPARS algorithm with global branch-swapping available in Felsenstein's PHYLIP Version 3.3.1 and the branch and bound algorithm of Hendy and Penny (1982), also available on PHYLIP 3.3.1.

To judge the relative stability of various parts of the most parsimonious trees, the data were subjected to "bootstrapping," again using PHYLIP 3.3.1. Here, nucleotides were sampled at random with replacement, and for each data set a new most parsimonious tree was constructed (using DNAPARS). The idea of bootstrapping is that nodes strongly supported by all of the data will also be supported by subsets of the data. Conversely, nodes weakly supported by all of the data will jump around when subsets of the data are used (Felsenstein, 1985). We used a total of 20 bootstrapped trees.

The distance analyses were based on  $K_{nuc}$  values following the formula of Hori (1975). Two methods for distance analysis were used, neither of which assume constant rates of evolution—the Fitch–Margoliash method (Fitch and Margoliash, 1967) also available on PHYLIP, and the distance Wagner procedure (Farris, 1972).

## RESULTS

The rapid sequencing method yielded a total of 488 bases for *S. gigantea*. Figure 1 shows the derived srRNA *S. gigantea* sequences aligned against those of *T. gondii* and *P. berghei*.

For the purposes of phylogenetic analysis, only the semiconserved regions were included, because the more variable regions represent "noise"

in the analysis. This left a total of 369 sites for analysis. The results of the comparative analysis of primary sequences are shown in Table 1.

The parsimony analysis yielded 2 equally parsimonious trees, each with 977 steps, that differed only in the relative placement of *Zea* and *Acanthamoeba*. A strict consensus tree for these 2 trees is shown in Figure 2. Here, *Sarcocystis* is monophyletic with *Toxoplasma*, and these 2 together are the sister-taxon of *Plasmodium*.

In order to gain some idea of the statistical robustness of the tree, the data were subjected to bootstrapping. The results are shown in Figure 3. Here, the monophyly of *Sarcocystis* and *Toxoplasma* was supported by all 20 bootstraps. Similarly, the monophyly of *Homo* and *Artemia* (Metazoa) was supported by all 20, as was the placement of the Metazoa as the sister-taxon to a clade consisting of *Sarcocystis*, *Toxoplasma*, *Plasmodium*, *Prorocentrum*, *Saccharomyces*, *Tetrahymena*, *Zea*, and *Acanthamoeba*. The placement of *Vairimorpha* as the sister-taxon to all other taxa used was evident in 19 of the 20 bootstraps, as was the placement of *Euglena* and *Trypanosoma* between *Vairimorpha* and the remaining taxa. By contrast, other nodes were weakly supported. Of particular interest here is the placement in the most parsimonious trees of *Plasmodium* as the sister-taxon to the *Sarcocystis*/*Toxoplasma* clade, a placement that was supported by only 10 of the 20 bootstraps. Figure 4 shows the maximum likelihood estimate of tree topology. This topology is identical to one of the most parsimonious trees.

The best-fit Fitch–Margoliash tree for the  $K_{nuc}$  distances had a standard deviation of 4.6% (Fig. 5). This tree is essentially identical in topology to the most parsimonious trees except that *Tetrahymena* is no longer the sister-taxon of *Saccharomyces*, and, of special interest here, *Plasmodium* is not the sister-taxon of the *Sarcocystis*/*Toxoplasma* clade.

The distance Wagner tree for the same set of data is shown in Figure 6. The topology of this tree is similar to the Fitch–Margoliash tree in showing monophyly of *Sarcocystis* and *Toxoplasma*, and also in placing *Plasmodium* outside the *Sarcocystis*/*Toxoplasma* clade.

## DISCUSSION

*Sarcocystis gigantea* is transmitted to sheep from cats. It causes the formation of macroscopic sarcocysts in the esophagus and tongue, but is otherwise nonpathogenic (Munday and Oben-

## A-SEQUENCE

						310	
Tg	GGAGAGGGAG	CCUGAGAAUC	GGCUACCACA	UCUAAGG-XG	GCAGCAGGCG	XXXXXAUUCC	AUUAC
Sg	GGAGAGGGAG	GCUGAGAAAU	AGCUACCACA	UCUAAGGAAAG	GCAGCAGGCG	CGUAAAUUAC	
Pb	GGAGAGGGAG	GCUGAGAAAU	AGCUACCACA	UCUAAGGAAAG	GCAGCAGGCG	CGUAAAUUAC	
Tg	CCAAUCCUGA	ACCAGGGAGG	-AGUGAC--G	AAAUAACAAC	ACUGGAAA--	UUUCUU-UCU	
Sg	CX-AUCCUGA	CUCAGGGAGG	UAGUGACAAG	AAAUAACAAC	ACUGGAAAUU	GUA-UU-UCU	
Pb	CCAAUUCUAA	AUAAGAGAGG	UAGUGACAAG	AAAUAACAAC	AUAAGGCCAA	AUUUUGG-UU	
Tg	-AGUGAUUGG	AAU					
Sg	-AGUGAUUGG	AAU					
Pb	UUAUAAUUGG	AAU					
		442					

## B-SEQUENCE

							690
Tg	GGUGAAAUUC	UUAGAUUUGU	UAAAGACG-A	CUACUGCGAA	AGCAUUUGCC	AAAGAUGUUU	
Sg	GGUGAAAUUC	UUAGAUUUGU	UAAAGACGAA	CUACUGCGAA	AGCAUUUGCC	AAAGAUGUUU	
Pb	GGUGAAAUUC	UUAGAUUUUC	UGGAGACAAA	CAACUGCGAA	AGCAUUUGCC	UAAAAUACUU	
Tg	UCAUUAUAUC	AGAACGAAAAG	UUAGGGGCGUC	GAAGACGAUC	-GAUACCGUC	GUAGUCUUAA	
Sg	UCAUUAUAUC	AGAACGAAAAG	UUAGGGGCGUC	GAAGACGAUC	AGAUAACCGUC	GUAGUCUUAA	
Pb	CCAUAUAUAUC	AGAACGAAAAG	UUAAGGGAGU	GAAGACGAUC	AGAUAACCGUC	GUAAUCUUAA	
Tg	CCAUAUAUAUC	UGCCGACUAG	AGAUAGGAAA	A-CGUCA---	-----	-----	
Sg	CCAUAUAUAUC	UGCCGACUAG	AGAUAGGAA-	AUGUCACA--	-----	-----	
Pb	CCAUAUAUAUC	UGCCGACUAG	GUUUUGGAUG	AAAAUUUUUA	AAUAAGAUUU	CCCUUCGGGG	
Tg	-----	--UGCUUGAC	U-UCUCCC--	CCUUAUGAGA	AAUC-AAAGUC		
Sg	-----	-UUG-UUGCU	UCUCCXXC-A	CCUUAUGAGA	AAUCAAAGUC		
Pb	AUUUCUUAGA	UUGCUUCCUU	CAGUACCUU-	----AUGAGA	AAUCAAAGUC		
						881	

## C-SEQUENCE

							1198
Tg	GUCUGCUGAU	GCCCUUAGAU	GUUCUGGGCU	AGCACGCGCG	CUACACUGAU	GCAUCCAACG	
Sg	GUCUG-UGAU	GCXCUXXXAU	GUUCUGGGCU	-GCACGCGCG	CUACACUGAU	GCAUCCAACA	
Pb	GUCUG-UGAU	GUCCUUAGAU	AUACUAGGCU	-GCACGCGUG	CUACACUGAU	AUGUAAAACG	
Tg	AGU---UUAU	A----ACC-U	UGGCCGA-UA	--GGUCU-AG	-----	-----	
Sg	XGUUGUAUUA	UXAAXACCUU	GGCCGAU---	AGGUUUA-GG	-----	-----	
Pb	AGU---GCUU	A----AAU-U	UA-UAUCU--	GUGCUUAGGU	GUUAAAGCCU	AUGUUUCAGU	
Tg	-----	-----	-----	AA-UCUUGUG	AGUAU--GU-	--CGU-GAUU	
Sg	-----	-----	-----	UAAUCUUUUG	AAGUAAUGCA	-UCGUUG---	
Pb	AUAUAUUUUU	CCUCCACUGA	AAAGUGUAGG	UAAUCUUUAU	CAAU-ACAU-	AUCGU-GAU-	
Tg	GGGGAUU---	--AUUGGCCA	UUAUUAAUCU	UUAAC-CCGA	AUGCCUAGUA		
Sg	-GGGAGACAU	UAUUUG-CAA	UUAUUAAUCU	UCAA-CCGA	AU-CCUAGUA		
Pb	GGGGAUAG-A	UUAUUUG-CAA	UUAUUAAUCU	UGAACGAGGA	AUGCCUAGUA		
						1349	

FIGURE 1. Sequence blocks of *Toxoplasma gondii* srRNA (Tg) aligned against those of *Sarcocystis gigantea* srRNA (Sg) and *Plasmodium berghei* srRNA (Pb). The numbering system is that of the *Escherichia coli* srRNA sequence of Brosius et al. (1981). Bases under the lines are those that were used for phylogenetic comparison. X denotes nucleotides of undetermined type.

dorf, 1984; Dubey et al., 1986). Although speciation within the genus appears to be well documented, the relationship of *Sarcocystis* and *Toxoplasma* has not been definitively estab-

lished. In order to investigate this relationship at the molecular level we have sequenced the semiconserved regions of the srRNA of a representative example of each genus.



TABLE I.  $K_{nuc}$  values based upon 396 semiconserved nucleotide sites for *Sarcocystis gigantea* and 15 other taxa.

	Hs	As	Sc	Tg	Dd	Tt	Sg	Zm	Tb	Eg	Pm	Pb	Ac	Vn	Hc	Ec
<i>Homo sapiens</i>	0															
<i>Artemia salina</i>	152	0														
<i>Saccharomyces cerevisiae</i>	238	258	0													
<i>Toxoplasma gondii</i>	271	318	174	0												
<i>Dictyostelium discoideum</i>	341	347	272	329	0											
<i>Tetrahymena thermophila</i>	339	384	216	256	346	0										
<i>Sarcocystis gigantea</i>	309	335	211	116	334	260	0									
<i>Zea mays</i>	223	258	124	157	268	257	186	0								
<i>Trypanosoma brucei</i>	494	528	499	498	449	560	520	442	0							
<i>Euglena gracilis</i>	452	464	414	431	454	478	466	386	434	0						
<i>Prorocentrum micans</i>	220	278	144	134	291	242	158	115	473	433	0					
<i>Plasmodium berghei</i>	344	390	227	245	357	336	269	224	558	492	178	0				
<i>Acanthamoeba castellanii</i>	238	281	148	194	265	264	206	107	474	415	120	233	0			
<i>Vairimorpha necatrix</i>	701	753	671	712	686	679	701	664	729	679	657	650	693	0		
<i>Halobacterium cutirubrus</i>	795	701	749	803	736	820	843	749	791	707	772	793	736	896	0	
<i>Escherichia coli</i>	784	788	767	822	763	850	845	782	727	697	763	780	754	842	518	0

srRNA is a cellular component that is good to use for the construction of evolutionary trees, as almost all organisms possess RNA, and the nucleotide sequences obtained can be stored and used for comparisons as new sequences become available. In addition, the rate of nucleotide substitutions, deletions, or insertions in various seg-

ments of the srRNA is not uniform; some areas are highly conserved, unchanged through millions of years of evolution, others are highly variable, and the remainder is semiconserved. Until recently, srRNA sequence comparisons required cloning of the gene before sequencing could begin. This was a time-consuming process,

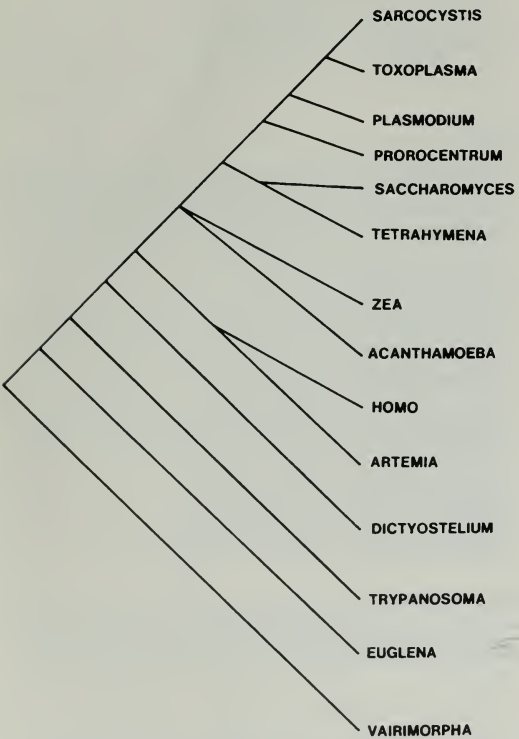


FIGURE 2. The consensus of the 2 most parsimonious trees found for the 14 taxa included. The trees were rooted using bacteria as outgroups. The most parsimonious trees found had a length of 977 steps.

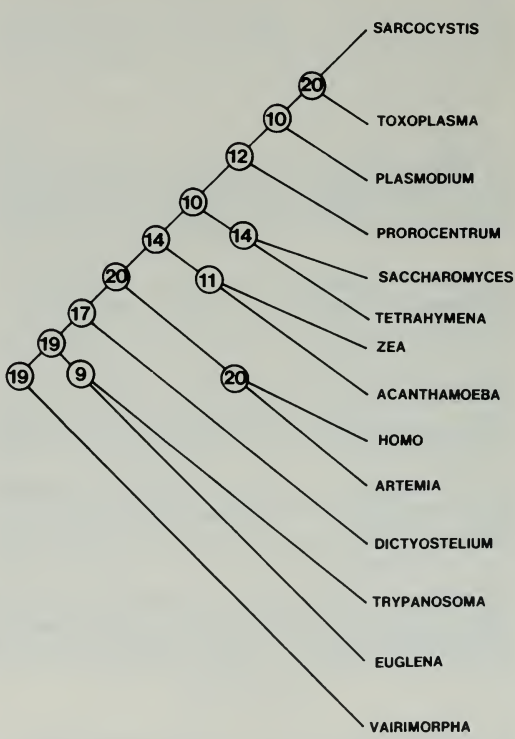


FIGURE 3. Results of bootstrapping the data that gave the trees in Figure 2. The values at the nodes indicate the number of times out of 20 that the node was supported by the bootstrap.

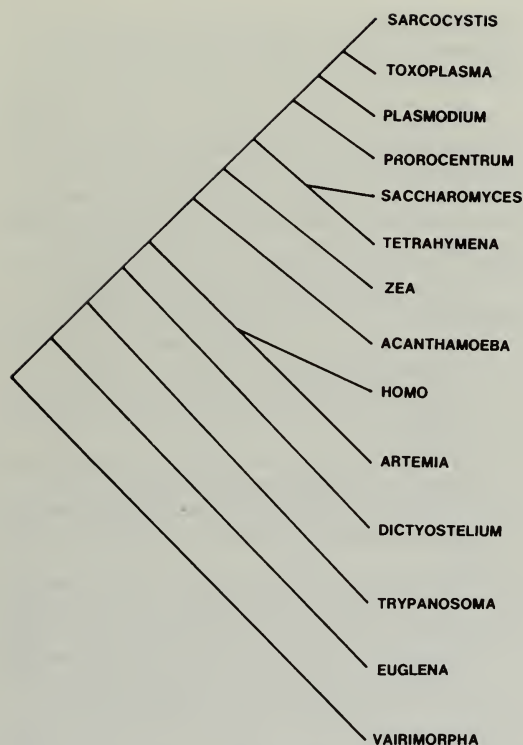


FIGURE 4. Maximum likelihood estimates of the relationship among the 14 taxa, rooted using bacteria as outgroups.

and is probably the major reason why only about 20 eukaryotic srRNA genes had been sequenced (Huysmans and De Wachter, 1985). However, the finding of Lane et al. (1985) that 3 conserved segments on the srRNA can be used as priming sites for reverse transcription and rapid sequencing of the semiconserved regions of srRNA will allow the inference of phylogenetic relationships based on srRNA sequence comparisons at a much faster rate than previously possible (Woese, 1987). In addition, Lane et al. (1985) have shown that the accuracy of the sequences obtained by this rapid nucleotide analysis of srRNA compares very favorably to that obtained with the much longer cloning and sequencing technique.

We compared srRNA sequence blocks of *S. gigantea* with those of 13 other eukaryotic taxa. Appropriate methods of phylogenetic analysis of sequence data have proved to be remarkably controversial (e.g., Felsenstein, 1982, 1984), so we therefore analysed this data set by 4 independent methods, none of which assume constant rates of evolution of the characters being used. By using such an approach, we hoped to

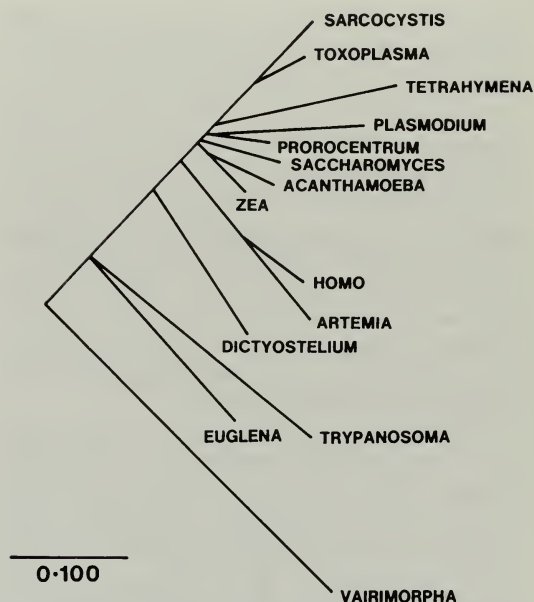


FIGURE 5. The best-fit Fitch-Margoliash tree for the 14 taxa, rooted using bacteria, for the  $K_{nuc}$  distances shown in Table I. Branch lengths are proportional to the amount of proposed evolutionary change along each branch. The standard deviation for this tree was 4.5%.

be able to distinguish spurious relationships from evolutionary relationships.

The overall topologies of the trees are in general agreement with each other, and indeed in agreement with other published analyses of the

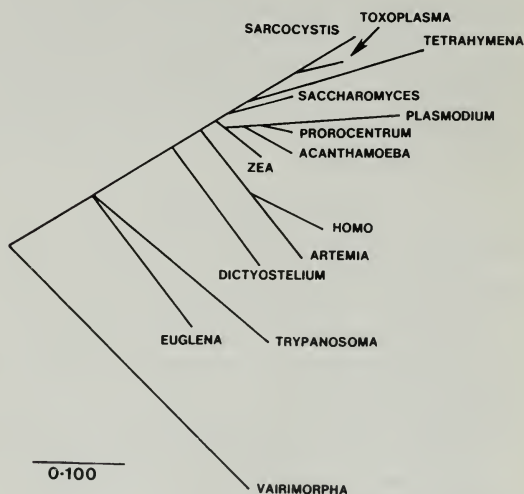


FIGURE 6. The tree produced from the distance Wagner procedure of Farris (1972) for the  $K_{nuc}$  distances in Table I. The branch lengths shown are proportional to the proposed amount of evolutionary change.



phylogenetic relationships among *Vairimorpha*, *Euglena*, *Trypanosoma*, *Dictyostelium*, metazoa, plants, fungi, and ciliates (Elwood et al., 1985; Vossbrinck et al., 1987). However, our placement of the dinoflagellate *Prorocentrum* differs from that of Herzog and Maroteaux (1986). Their analysis placed the origin of *Prorocentrum* near the *Dictyostelium*/metazoa node, whereas all of our analyses place *Prorocentrum* closer to *Saccharomyces* (which they included in their analysis). We are unable to establish why this discrepancy exists because Herzog and Maroteaux (1986) did not indicate which parts of the sequence they used for analysis.

With regard to the placement of *Sarcocystis*, all 4 analyses agree in showing it to be monophyletic with *Toxoplasma*. It therefore seems certain that these taxa are monophyletic. The position of the third apicomplexan, *Plasmodium*, with regard to the remaining 2 was left largely unresolved by our analysis, as indeed it was in our earlier analysis that did not include *Sarcocystis* (Johnson et al., 1987). More such analyses will be required to determine the phylogenetic validity of the organisms presently included in the phylum Apicomplexa.

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APPENDIX. Data base used for the construction of trees. Legend is as follows: H.s. *Homo sapiens*, M.m. *Mus musculus*, R.n. *Rattus norvegicus*, O.c. *Oryctolagus cuniculus*, X.l. *Xenopus laevis*, A.s. *Artemia salina*, S.c. *Saccharomyces cerevisiae*, T.g. *Toxoplasma gondii*, S.g. *Sarcocystis gigantea*, N.c. *Neurospora crassa*, D.d. *Dictyostelium discoideum*, O.n. *Oxytricha nova*, S.p. *Stylonychia pustulata*, T.t. *Tetrahymena thermophila*, O.s. *Oryza sativa*, Z.m. *Zea mays*, G.m. *Glycine max*, T.b. *Trypanosoma brucei*, E.g. *Euglena gracilis*, P.t. *Paramecium tetraurelia*, E.a. *Euplotes aediculatus*, P.m. *Prorocentrum micans*, P.b. *Plasmodium berghei*, A.c. *Acanthamoeba castellanii*, V.n. *Vairimorpha necatrix*, H.c. *Halobacterium cutirubrus*, E.c. *Escherichia coli*. The large inserts in T.b., A.c., and P.b. in sequence block B, and P.b. and A.c. in sequence block C, are not aligned. Bases under the lines are those that were used for phylogenetic comparison. X denotes nucleotides of undetermined type.





## B-SEQUENCE

H.s.	GGUGAAAUUCUUGGACCGGCGCAAGACGGACCAGAGCGAAAGCA---UUUGCCAAGAAUGUUUUAUUAUUC
M.m.	GGUGAAAUUCUUGGACCGGCGCAAGACGGACCAGAGCGAAAGCA---UUUGCCAAGAAUGUUUUAUUAUUC
R.n.	GGUGAAAUUCUUGGACCGGCGCAAGACGAACAGAGCGAAAGCA---UUUGCCAAGAAUGUUUUAUUAUUC
O.c.	GGUGAAAUUCUUGGACCGGCGCAAGACGGACCAGAGCGAAAGCA---UUUGCCAAGAAUGUUUUAUUAUUC
X.l.	GGUGAAAUUCUUGGACCGGCGCAAGACGAACCAAAGCGAAAGCA---UUUGCCAAGAAUGUUUUAUUAUUC
A.s.	GGUGAAAUUCUUGGACCGUCGCAAGACGAACAACUGCGAACAAG---UUUGCCAAGAAUGUUUUAUUAUUC
S.c.	GGUGAAAUUCUUGGAUUUAUUGAAGACUAAACUACUGCGAAAGCG---UUUGCCAAGGACGUUUUCGUUAUUC
T.g.	GGUGAAAUUCUAGAUUUUGUUAAAGACG-ACUACUGCGAAAGCA---UUUGCCAAGAAUGUUUUAUUAUUC
S.g.	GGUGAAAUUCUAGAUUUUGUUAAAGACGAACUACUGCGAAAGCA---UUUGCCAAGAAUGUUUUAUUAUUC
N.c.	GGUGAAAUUCUUGGAUUUAUUGAAGACUAAACUACUGCGAAAGCA---UUUGCCAAGGAUGUUUUAUUAUUC
D.d.	GGUGAAAUUCGUUGACCCUAUCAAGAUGAACUUCUGCGAAAGCA---UUCACCAAAUACUCCCAUUAUUC
O.n.	GGUGAAAUUCUGGAUUUGUUAAAGACUAAACUUAUGCGAAAGCA---UUUGCCAAGGAUGUUUUAUUAUUC
S.p.	GGUGAAAUUCUCAGAUUUUGUUAAAGACUAAACUUAUGCGAAAGCA---UUUGCCAAGGAUGUUUUAUUAUUC
T.t.	GGUGAAAUUCUUGGAUUUAUUAAGGACUAAACUUAUGCGAAAGAC---UUUGCCAAGAAUGUUUUAUUAUUC
O.s.	GGUGAAAUUCUUGGAUUUAUUGAAGACGAACAACUGCGAAAGCA---UUUGCCAAGGAUGUUUUAUUAUUC
Z.m.	GGUGAAAUUCUUGGAUUUAUUGAAGACGAACAACUGCGAAAGCA---UUUGCCAAGGAUGUUUUAUUAUUC
G.m.	GGUGAAAUUCUUGGAUUUAUUGAAGACGAACAACUGCGAAAGCA---UUUGCCAAGGAUGUUUUAUUAUUC
T.b.	GGUGAAAUUCUAGACCGCACCAAGACGAACUACAGCGAAGGCA---UUCUUCUAGGAUACCUUCCUCAUUC
E.g.	GGUGAAAUUCUAGAUUCGCGCAGAUCCACUGCAGCGAAGGCG---UUCUGCAAGUGCAGCUGCCGUCGAUC
P.t.	GGUGAAAUUCUUGGAUUUAUUAAGACUAAACUUAUGCGAAAGCA---UUUGCCAAGGAUGUUUUAUUAUUC
E.a.	GGUGAAAUUCUUGGAUUUAUUAAGACUAAACUUAUGCGAAAGCAUCGUUUUGCCAUGAUUUUUAUUAUUC
P.m.	GGUGAAAUUCUGGAUUUGUUAAAGACGGACUACUGCGAAAGCA---UUUGCCAAGGAUGUUUUAUUGAUC
P.b.	GGUGAAAUUCUAGAUUUUCUGGAGACAAACAACUGCGAAAGCA---UUUGCCUAAAUAACUCCAUAUUAUC
A.c.	GGUGAAAUUCUUGGAUUUAUGAAGAUUAACUUCUGCGAAAGCA---UCUGCCAAGGAUGUUUUAUUAUUC
V.n.	GAUGAAAUGUGACGACCCUGACUGGACGAACAGAAAGCGAAAGCU---GUACACUUGUAUGUAUUUUUGAAG
H.c.	AGUGAAAUCCUGUAUUCUGGACGAGACCGCGUGGCGAAAGCG---CCUCAGGAGAACGGAUCCGACAGUG
E.c.	GGUGAAAUGCGUAGAGAUUCUGGAGGAUACCGGUGGCGAAGGCG---GCCCCUGGACGAAGACUGACGCU
<hr/>	
H.s.	AA-GAACGAAAGUCGGAGGUUCGAAGACGAUCAGAUACCGUCGUAGUCCG--ACCAUAAACGAUGCCGACC
M.m.	AA-GAACGAAAGUCGGAGGUUCGAAGACGAUCAGAUACCGUCGUAGUCCG--ACCAUAAACGAUGCCGACU
R.n.	AA-GAACGAAAGUCGGAGGUUCGAAGACGAUCAGAUACCGUCGUAGUCCG--ACCAUAAACGAUGCCGACU
O.c.	AA-GAACGAAAGUCGGAGGUUUGAAGACGAUCAGAUACCGUCGUAGUCCG--ACCACAAACGAUGCCGACU
X.l.	AA-GAACGAAAGUCGGAGGUUCGAAGACGAUCAGAUACCGUCGUAGUCCG--ACCAUAAACGAUGCCGACU
A.s.	AA-GAACGAAAGUUGAGAGGUUCGAAGGCGAUCAGAUACCGCCUAGUUCUA--ACCAUAAACGAUGCCAAAC
S.c.	AA-GAACGAAAGUUGAGGGGUCGAAGACGAUCUGAUACCGUCGUAGUCUUA--ACCAUAAACUAGCCGACU
T.g.	AA-GAACGAAAGUUGAGGGGUCGAAGACGAUC-GAUACCGUCGUAGUCUUA--ACCAUAAACUAGCCGACU
S.g.	AA-GAACGAAAGUUGAGGGGUCGAAGACGAUCAGAUACCGUCGUAGUCUUA--ACCAUAAACUAGCCGACU
N.c.	AG-GAACGAAAGUUGAGGGGUCGAAGACGAUCAGAUACCGCUGUAGUCUUA--ACCAUAAACUAGCCGAUU
D.d.	AA-GAACGAAAGUUGAGGGGUCGAAGACGAUCAGAUACCGUCGUAGUCCAA--ACUUAUAAACUAGUCGACC
O.n.	AA-GAACGAAAGUUGAGGGGUCGAAGACGAUCAGAUACCGUCCUAGUCUUA--ACCAUAAACUAGCCGACU
S.p.	AA-GAACGAAAGUUGAGGGGUCGAAGACGAUCAGAUACCGUCCUAGUCUUA--ACCAUAAACUAGCCGACU
T.t.	AA-GAACGAAAGUUGAGGGGUCGAAGACGAUCAGAUACCGUCGUAGUCUUA--ACUUAUAAACUAGCCGACU
O.s.	AA-GAACGAAAGUUGAGGGGUCGAAGACGAUCAGAUACCGUCCUAGUCUUA--ACCAUAAACGAUGCCGACC
Z.m.	AA-GAACGAAAGUUGAGGGGUCGAAGACGAUCAGAUACCGUCCUAGUCUUA--ACCAUAAACGAUGCCGACC
G.m.	AA-GAACGAAAGUUGAGGGGUCGAAGACGAUCAGAUACCGUCCUAGUCUUA--ACCAUAAACGAUGCCGACC
T.b.	AA-GAACGAAAGUUGAGGGGUCGAAGACGAUCAGAUACCGUCCUAGUCUUA--ACCAUAAACGAUGCCGACC
E.g.	AA-GAAUGAGAGUUCGGGGAGCAAAGAUGAUCAGACACCGUCGUAGUCCGGCCACUGUAAACGAUGCCGGCC
P.t.	AA-GAACGAAAGUUGAGGGGUCGAAGACGAUCAGAUACCGUCGUAGUCUUA--ACUUAUAAACUAGCCGACU
E.a.	AUUGAACGAAAGUUGAGGGGUCGAAGACGAUCAGAUACCGUCCUAGUCUUA--ACCAUAAACGUUGCCGACU
P.m.	AA-GAACGAAAGUUGAGGGGUCGAAGACGAUCAGAUACCGUCCUAGUCUUA--ACCAUAAACGAUGCCGACU
P.b.	AA-GAACGAAAGUUAAGGGAGUGAAGACGAUCAGAUACCGUCGUAAUCUUA--ACCAUAAACGAUGCCGACU
A.c.	AA-GAACGAAAGUUGAGGGGUCGAAGACGAUCAGAUACCGUCGUAGUCUUA--ACCAUAAACGAUGCCGACC
V.n.	AA-GGACGUAAGCUGGAGGAGCGAAGAUAUAGAUACCAUUGUAGUCCAA--GCAGUAAACUAGCCGACG
H.c.	AG-GGACGAAAGCUAGGGUCUCGAACCGGAUUAAGAUACCGGGUAGUCCAA--GCUGUAAACGAUGCCGCU
E.c.	AG-GUGCGAAAGCGUGGGGAGCAAACAGGAUAUAGAUACCGUGUAGUCCAA--GCCGUAAACGAUGUCGACU



B-SEQUENCE (CONTINUED)

H.s. GGCGAUGCGGCG-GCGUUAU-----  
M.m. GGCGAUGCGGCG-GCGUUAU-----  
R.n. GGCGAUGCGGCG-GCGUUAU-----  
O.c. GGCGAUGCGGCG-GCGUUAU-----  
X.l. AGCGAUCCGGCG-GCGUUAU-----  
A.s. AGCGAUCCGGCG-GCGUUAU-----  
S.c. AGGGAUCGGGUG-GUGUUUU-----  
T.g. AGAGAUAGGAA-AA-CGUCA-----  
S.g. AGAGAUAGGAA--AUGUCACA-----  
N.c. AGGGAUCGGACG-GUGUUAU-----  
D.d. AGGGAUCGGUUA-AAAUUUU-----  
O.n. AGGGAUCGGAGG-CG--CGC-----  
S.p. AGGGAUCAGAGG-CG--CGC-----  
T.t. CGGGAUCGGCUG-GAAUAAU-----  
O.s. AGGGAUCGGCGG-AUGUUGC-----  
Z.m. AGGGAUCAGCGG--UGUUAC-----  
G.m. AGGGAUCAGCGG-AUGUUGC-----  
T.b. AUGAAUUGGGGA-ACAUCAUUGGGUGCCCUGUGGCGGCCUUUUGUGCCGACCCUCGGCCCCAAUUAUUUA  
E.g. AGGCCUUGGCAG-AGCAAGAA-----  
P.t. CGAGAUCCGGAAG-GGUAAUAUAUA-----  
E.a. AGGGAUCGGAGGGCGUGCACAUA-----  
P.m. AGAGAUUGGAGG-UCGUUAUCUAUA-----  
P.b. AGGUUUUGGAUG-AAAAUUU-----  
A.c. AGCGAUUAGGAG-ACGUUGAAUACAAACACCACCAUCGGCGCGGUCGUCCUUGGCGUCUGUCCCUUUAAC  
V.n. AUGUGAUUGAU-A-----  
H.c. AGGUGUGGCGCA-GGCUACG-----  
E.c. UGGAGGUUGUGC-CCU-----

H.s. -----UCCCAUGACC  
M.m. -----UCCCAUGACC  
R.n. -----UCCCAUGACC  
O.c. -----UCCCAUGACC  
X.l. -----UCCCAUGACC  
A.s. -----UUGAAUGACU  
S.c. -----UUUAAUGACC  
T.g. -----UGCUUGACU  
S.g. -----UUGUUG-CUU  
N.c. -----UUU-UUGACC  
D.d. -----UUC-AAAAUU  
O.n. -----UUUAUC--CG  
S.p. -----UUUAUC--CG  
T.t. -----GUCCAGUCGG  
O.s. -----UUUAUGGACU  
Z.m. -----UAAUAGGACC  
G.m. -----UUUUAGGACU  
T.b. UCAAUUUACGUGCCUAUUCUACACCCGGUUCUCCUUCUUGAGGUUCUUCGCGGUAUUUUUACG-GGAAUA  
E.g. -----UCC-UAGACU  
P.t. -----GUCCCUUUCGG  
E.a. -----CCGCCUUCGG  
P.m. -----CGACUCCUUC  
P.b. -----UAAUAAGAUUUCCCUUCGCGGAUUUCUAGAUUGCUUCCUUC  
A.c. GGGGGCAGGCGGAGGGCGGUUAGCCCGGUGGCACCGUGAAUG-----ACUCCCCUAG  
V.n. -----UUUA  
H.c. -----AGCCUGCGCU  
E.c. -----UGAGGCGUGG

## B-SEQUENCE (CONTINUED)

H.s.	CGCCGGG----	C-AGCUUCC-----	GGGAAACCA-AAGUC
M.m.	CGCCGGG----	C-AGCUUCC-----	GGGAAACCA-AAGUC
R.n.	CGCCGGG----	C-AGCUUCC-----	GGGAAACCA-AAGUC
O.c.	CGACGGG----	C-AGCUUCC-----	GGGAAACCA-AAGUC
X.l.	CGCCGAG----	C-AGCUUCC-----	GGGAAACCA-AAGUC
A.s.	CCGCGGG----	C-AGCUUCC-----	GGGAAACCA-AAGUC
S.c.	CACUCGG----	U-ACCUUAC-----	GAGAAAUCA-AAGUC
T.g.	-UCUCC-----	CCUUAU-----	GAGAAAUCA-AAGUC
S.g.	CUCCXCC-----	ACCUUAU-----	GAGAAAUCA-AAGUC
N.c.	CGUUCGG----	C-ACCUUAC-----	GAUAAAUCA-AAAUG
D.d.	UAAUCGG----	C-ACCUUGU-----	GAGAAAUCAUGAGUG
O.n.	CCUUCGG----	C-ACCUUAU-----	GAGAAAUCA-AAGUC
S.p.	CCUUUGG----	C-ACCUUAU-----	GAGAAAUCA-AAGUC
T.t.	CACCGUA-----	U-----GA-----	GAGAAGCUA-AAGUC
O.s.	CCGCCGG----	C-ACCUUAU-----	GAGAAAUCA-AAGUC
Z.m.	CCGCUUG-----	CCACCUUAU-----	GAGAAAUCA-AAGUC
G.m.	CCGCUUG-----	C-ACCUUAU-----	GAGAAAUCA-AAGUC
T.b.	UCCUCAG-----	C-ACGUUUCUUACUUCUUCACGCGAAAGCUUGGAGG	
E.g.	CUGUCAGGGCCAC-UCCUCCACAC-----	ACGAGAAAUCCACAGCC	
P.t.	CAUCGUA-----	A-----	GAGAAAUCA-AAGUC
E.a.	CACCUUA-----	C-----	GAGAAAUCA-AAGUC
P.m.	AGCACC-----	U-----AU-----	GAGAAAUCA-AAGUC
P.b.	AGUACC-----	U-----AU-----	GAGAAAUCA-AAGUC
A.c.	CAGCUUG-----		GAGAAAUCAUAAGUC
V.n.	--AUUGUA-----	U-UAGAUGA-----	UAGAAAUUU-GAGUU
H.c.	GUGCCGU-----	AGGGAAG-----	CCGAGAAGCGGACCG
E.c.	CUUCCGG-----	AGCUAAC-----	GCGUUAAGUCGACCG



## C-SEQUENCE

H.s. GUCUG-UGAUGCCCUUAG-AUGUCCGGGGCU-GCACGCGCGCUACACUGA---CUGGCUACGCGUG-----  
M.m. GUCUG-UGAUGCCCUUAG-AUGUCCGGGGCU-GCACGCGCGCUACACUGA---CUGGCUACGCGUG-----  
R.n. GUCUG-UGAUGCCCUUAG-AUGUCCGGGGCU-GCACGCGCGCUACACUGA---CUGGCUACGCGUG-----  
O.c. GUCUG-UGAUGCCCUUAG-AUGUCCGGU-CG-GCACGCGCGCUACACUGA---CUGGCUACGCGUG-----  
X.l. GUCUG-UGAUGCCCUUAG-AUGUCCGGGGCU-GCACGCGCGCUACACUGA---ACGGAUCAGCGUG-----  
A.s. GUCUG-UGAUGCCCUUAG-AUGUCCGGGGCU-GCACGCGCGCUACACUGG---AAGAAUCAGCGCG-----  
S.c. GUCUG-UGAUGCCCUUAGAACGUUCUGGGCC-GCACGCGCGCUACACUGA---CGGAGCCAGCGAG-----  
T.g. GUCUGUGAUGCCCUUAG-AUGUUCUGGGCUAGCACGCGCGCUACACUGA---UGCAUCCAACGAG-----  
S.g. GUCUG-UGAUGCXCUXXX-AUGUUCUGGGCU-GCACGCGCGCUACACUGA---UGCAUCCAACAX-----  
N.c. GUCUG-UGAUGCCCUUAG-AUGUUCUGGGCC-GCACGCGCGCUACACUGA---CACAGCCAGCGAG-----  
D.d. GUCUG-UGAUGCCCUUAG-AUACCUUGGGCC-GCACGCGCGCUACAAUGU---AGGAAACAAAAAG-----  
O.n. GUCUG-UGAUGCCCUUAG-AUGUCCUGGGCC-GCACGCGUGCUACACUGA---CGCAUACAGCGAG-----  
S.p. GUCUG-UGAUGCCCUUAG-AUGUCCUGGGCC-GCACGCGUGCUACACUGA---CGCAUACAGCGAG-----  
T.t. GUCUG-UGAUGCCCUUAG-ACGUGCUCGGCC-GCACGCGCGUUAACAAUGA---CUGGCGCAAAAAG-----  
O.s. GUCUG-UGAUGCCCUUAG-AUGUUCUGGGC-GCACGCGCGCUACACUGA---UGUAUCCAACGAG-----  
Z.m. GUCUG-UGAUGCCCUUAG-AUGUUCUGGGCC-GCACGCGCGCUACACUGA---UGUAUCCAACGAG-----  
G.m. GUCUG-UGAUGCCCUUAG-AUGUUCUGGGCC-GCACGCGCGCUACACUGA---UGUAUUAACGAG-----  
T.b. GUCUG-UGAUGCUCCUCA-AUGUUCUGGGCG-ACACGCGCACUACAAUGUCAGUGAGAACAA-GAG--UCCG  
E.g. GUCUG-UGAUGCUCCAG-AUGUCCUGGGCC-GCACGCGCACUACAUUGU---CACAGUGAAGGUGUCGACA  
P.t. GUCUG-UGAUGCCCUUAG-ACGUCCUGGGCC-GCACGCGCGCUACACUGA---CACGUUCAGCGAGCU---  
E.ä. GUCUG-UGAUGCCCUUAG-AUGUCCUGGGCC-GCACGCGUGCUACACUGA---UACGUACAACGAGGU---  
P.m. GUCUG-UGAUGCCCUUAG-AUGUUCUGGGCU-GCACGCGCGCUACACUGA---UGCGUUAACGAG-----  
P.b. GUCUG-UGAUGUCCUAG-AUAUACUAGGCU-GCACGCGUGCUACACUGA---UAUGUAAAACGAG-----  
A.c. GUCUG-UGAUGCCCUUAG-AUGUUCUGGGCC-GCACGCGCGCUACACUGA---UUAUCCAACGAG-----  
V.n. GUCCG-UUAUGCCCUUAG-ACAUUUUGGGCU-GCACGCGCAAUACAAU-----  
H.c. GUCAG-U-AUGCCCGGAA--UGGGCUGGGCA-ACACGCGGGCUACAAUGG---UCGAGACAAUGGG-----  
E.c. GUCAU-C-AUGGCCCUGA--CGACCAGGGCU-ACACACGUGCUACAAUGG---CGCAUACAAAGAG-----

H.s. -----U---GCCUA---CCC-UACGCCGG-----  
M.m. -----U---GCCUA---CCC-UGCGCCGG-----  
R.n. -----U---GCCUA---CCC-UACGCCGG-----  
O.c. -----U---GCUUA---CCC-UACGCCGG-----  
X.l. -----U---GUCUA---CCC-UGCGCCGA-----  
A.s. -----U---CCU-----CCC-UGU-CCGA-----  
S.c. -----U---CUA-----ACC-UUGGCCGA-----  
T.g. -----U---UUAUA---ACC-UUGGCCGA---UA-----  
S.g. -----GUUGUAUUAUXAAXACC-UUGGCCGAU-----  
N.c. -----U---ACU-----CCC-UUGGCCGG-----  
D.d. -----G---C-----UCC-UGGUCCGG-----  
O.n. -----U---ACU-----UCCCAGCUCCGA-----  
S.p. -----U---ACU-----UCCCAGCUCCGA-----  
T.t. -----U---AUU-----UCC-UGUCCUGG-----  
O.s. -----U---AUUA---GCC-UGGUCCGA-----  
Z.m. -----U---AUUA---GCC-UUGGCCGA-----  
G.m. -----U---CUAUA---GCC-UUGGCCGA-----  
T.b. AGCGGCACUUCACAAUGUCG-----CUC-CCGCUUGAUCAA-----  
E.g. UGCCCCAC---UCCG---GUGGG-----CCC-UGGCCUGA-----  
P.t. -----U---AUU-----UAC-CUGUCCCGA-----  
E.a. -----GUUAUUGU---ACU-----UGU-ACAUCAUAGCUGCUCC-----  
P.m. -----U---UUAUG---ACC-UUG-CCCAG-----  
P.b. -----U---GCUUA---AAU-UUA-UAUCU-----  
A.c. -----U---CCGCU---UCA-AUCGAGGCGGAUGCCGUUGGGGUCAAACCCAACUGUGUC-----  
V.n. -----AGUA-----  
H.c. -----A---AGCCA-----CUCCGA-----  
E.c. -----A---AGCGA-----CCUCGC-----

## APPENDIX p.6.

## C-SEQUENCE (CONTINUED)

H.s.	-----CA-GGCGC-GGG-----
M.m.	-----CA-GGCGC-GGG-----
R.n.	-----CA-GGCGC-GGG-----
O.c.	-----CA-GGC-C-GG-----
X.l.	-----CA-GGUGC-GGG-----
A.s.	-----GA-GGACC-GGG-----
S.c.	-----GA-GGUCU-UGG-----
T.g.	-----GGUCU-AG-----
S.g.	-----AG-GUUUA-GG-----
N.c.	-----AA-UGUCC-GGG-----
D.d.	-----AA-GGAUU-GGG-----
O.n.	-----GA-GGCAGCUGG-----
S.p.	-----GA-GGCAGCUGG-----
T.t.	-----GAAGGUAC-GGG-----
O.s.	-----CA-GGCCC-GGG-----
Z.m.	-----CA-GGCC-GG-----
G.m.	-----CA-GGUCC-GGG-----
T.b.	-----AA-GAGCG-GGG-----
E.g.	-----AG-AGGCU-GGG-----
P.t.	-----AAGGGCACGGG-----
E.a.	-----GAAAUAGACGAGCU-----
P.m.	-----UA-GGGUUUGGG-----
P.b.	-----GUG-GCUUAGGUGUUAAAGCCUAGUUUUCAGUAUUAUUUUUCCUC
A.c.	GCUGUCCUCGAUCGCGCCUGGGCCGAU-AGGUCCGGG-----
V.n.	-----UA-UAAUC-UU-----
H.c.	-----GA-GGAGG-CGCU-----
E.c.	-----GA-GAGCA-AGCG-----
H.s.	-----UAACCCGUUGAACCCCAUU---CGU-GAU-GGGGAUCG-GGGAUUG-CAAUUAUUCCC
M.m.	-----UAACCCGUUGAACCCCAUU---CGU-GAU-GGGGAUCG-GGGAUUG-CAAUUAUUCCC
R.n.	-----UAACCCGUUGAACCCCAUU---CGU-GAU-GGGGAUCG-GGGAUUG-CAAUUAUUCCC
O.c.	-----UAACCCGUUGAACCCCAUU---CGU-GAU-AGGGAUCG-GGGAUUG-CAAUUAUUCCC
X.l.	-----UAACCCGUGAACCCCGUU---CGU-GAU-AGGGAUCG-GGGAUUG-CAAUUAUUCCC
A.s.	-----UAA-CCGCUGAACCUUUC---CGU-GGU-UGGGAUUG-GGGACUG-CAAGGAUCCC-
S.c.	-----UAAUCUUGGAAACUCCGU---CGU-GCU-GGGGAUAG-AGCAUUG-UAAUUAUUGCU
T.g.	-----AA-UCUUGUGAGUAU---GU---CGU-GAUUGGGGAUU---AUUGGCCAUUUAUUAU
S.g.	-----UAAUCUUUUGAAGUAAUGCA-UCGU-UG---GGGAGACAUUAUUG-CAAUUAUUAU
N.c.	-----UAAUCUUGUUAACUGUGU---CGU-GCU-GGGGAUAG-AGCAUUG-CAAUUAUUGCU
D.d.	-----UAAUCAUUUGAAUUUCCUA---CGU-AAC-UGGGCUUG-AUCUUUC-UAAUUUAUUGAU
O.n.	-----UAAUC---AGCAUAUUGCGU---CGU-GAU-GGGGAUAG-AUCUUUG-GAAUUAUAGAU
S.p.	-----UAAUC---AGCAUAUUGCGU---CGU-GAU-GGGGAUAG-AUCUUUG-GAAUUAUAGAU
T.t.	-----UAAUCUUAUUAUACCAGU---CGU-GUU-AGGGAUAG-UUCUUUG-GAAUUGUGGAU
O.s.	-----UAAUCUUGGGAAUUUCAU---CGU-GAU-GGGGAUAG-AUCAUUG-CAAUUGUUGGU
Z.m.	-----UAAUCUUGGGAAUUUCAUG---CGU-GAU-GGGGAUAG-AUCAUUG-CAAUUGUUGGU
G.m.	-----UAAUCUU-UGAAAUUUCAU---CGU-GAU-GGGGAUAG-AUCAUUG-CAAUUGUUGGU
T.b.	-----AAACCA-CGGAUACAGUAG---ACCCACU-UGGGACCG-AGUAUUG-CAAUUAUUGGU
E.g.	-----AAAUCCUGCAGCCUGUGA---CGU-ACU-GGGGAUAG-AUGGUUG-CAACUGUCUGC
P.t.	-----AAAUUCUUGUAGGACGUGU---CGU-GCU-GGGGAUAG-AUCUUUG-CAAUUAUAGAU
E.a.	-----UAAUCUUAUAAAUACGUU---CGU-GCU-GGGGAUAG-AUCGUUG-AAAUUAUGAAU
P.m.	-----UAAUCUUUUUAAAUACGCAU---CGU-GAU-GGGGAUAG-AUUAUUG-CAAUUAUUAU
P.b.	ACUGAAAAGUGUAGGUAAUCUUUAUCAUACA-UACGU-GAU-GGGGAUAG-AUUAUUG-CAAUUAUUAU
A.c.	-----UAAUCUUGGCAAUUUUAU---CGU-GCU-GGGGAUAG-AUCAUUG-UAAUUAUUGAU
V.n.	-----UAU---GGGAUAA-UAAUUUG-UAAGAG-AUAU
H.c.	-----AAU-CUCCUAAACUCGAU---CGU-AGU-UCGGAUUG-AGGGCUG-AAACUCGCCCU
E.c.	-----GAC-CUCAUAAAGUGCGU---CGU-AGU-CCGGAUUG-GAGUCUG-CAACUCGACUC



## C-SEQUENCE (CONTINUED)

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H.s.	C-AUGAACGAGGAAUUCCCAGUA
M.m.	C-AUGAACGAGGAAUUCCCAGUA
R.n.	C-AUGAACGAGGAAUUCCCAGUA
O.c.	C-AUGAACGAGGAAUUCCCAGUA
X.l.	C-AUGAACGAGGAAUUCCCAGUA
A.s.	C-AUGAACGAGGAAUCCCUAGUA
S.c.	C-UUCAACGAGGAAUCCCUAGUA
T.g.	C-UUUAAC-CCGAAUGCCUAGUA
S.g.	C-UUCAAA-CGGAAU-CCUAGUA
N.c.	C-UUCAACGAGGAAUCCCUAGUA
D.d.	C-AUAAACGAGGAAUCCCUUGUA
O.n.	C-UUGAACGAGGAAUCCCUAGUA
S.p.	C-UUGAACGAGGAAUCCCUAGUA
T.t.	C-UUGAACGAGGAAUUCUAGUA
O.s.	C-UUCAACGAGGAAUGCCUAGUA
Z.m.	C-UUCAACGAGGAAUGCCUAGUA
G.m.	C-UUCAACGAGGAAUCCCUAGUA
T.b.	CGCGCAACGAGGAAUGUCUCGUA
E.g.	C-UUGAACGUGGAAUGCCUAGUA
P.t.	C-UUGAACGAGGAAUCCCUUGUA
E.a.	C-UUGAAGGUGGAAUCCCUAGUA
P.m.	C-UUCAACGAGGAAUCCCUAGUA
P.b.	C-UUGAACGAGGAAUGCCUAGUA
A.c.	C-UUCAACGAGGAAUCCCUAGUA
V.n.	--UUGAACUUGGAAUUGCUAGUA
H.c.	C-AUGAAGCUGGAUUCGGUAGUA
E.c.	C-AUGAAGUCGGAUUCGCUAGUA

**PSEUDOLERNAEOPODINA SYNAPHOBRANCHI N. GEN., N. SP.  
(COPEPODA: LERNAEOPODIDAE) FROM THE EYE OF  
SYNAPHOBRANCHUS KAUPI JOHNSTON, 1862, IN THE  
NORTHWEST ATLANTIC OCEAN**

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**ABSTRACT:** A new genus of parasitic copepod from the family Lernaeopodidae, *Pseudolernaepodina synaphobranchi*, is described from a single specimen found on the eye of the demersal eel, *Synaphobranchus kaupi*. The specimen exhibits a mixture of morphological features found in 3 other lernaepodid genera; *Clavellistes*, *Lernaepodina*, and *Pseudocharopinus*. Distinguishing morphological characters of the new genus include a nonprehensile second antenna armed at the apex of the endopod with 1 degenerate hook and 1 attenuated process, an absence of posterior processes, and a seta at the base of the lateral exopod of the first maxilla. *Pseudolernaepodina synaphobranchi* is tentatively aligned with the *Charopinus* group of the Lernaeopodidae, but is considered an aberrant genus, probably having evolved separately from other groups of genera in the family.

During a research cruise of the R/V Oceanus off the coast of New Jersey in March 1987, a single specimen of a lernaepodid copepod was found attached to the cornea of *Synaphobranchus kaupi*, a demersal eel. When examined, the copepod exhibited features that could not be accommodated in any presently known genus of the Lernaeopodidae. The specimen, *Pseudolernaepodina synaphobranchi* n. gen., n. sp. is herein described and figured, and its phylogeny discussed.

**MATERIALS AND METHODS**

The host, *Synaphobranchus kaupi* (38.6 cm TL) was collected from 1,975 m of water off the New Jersey coast on 16 March 1987. The gear used for host capture was a modified Gulf of Mexico shrimp trawl towed on bottom. The parasite and a portion of the infested eye were removed from the host and fixed and stored in 70% ethanol. After dissection from eye tissues, the copepod was mounted whole in 85% lactic acid and cleared for examination of fine structures. Appendages were viewed at magnifications of up to 950× under phase-contrast microscopy. Drawings were made with the aid of a drawing tube. Terminology follows that of Kabata (1979).

***Pseudolernaepodina synaphobranchi*  
n. gen., n. sp.  
(Figs. 1-7)**

*Host:* *Synaphobranchus kaupi* Johnston, 1862.

*Site of infestation:* Bulla embedded in cornea of eye.

*Locality:* Off New Jersey (39°46'N, 70°40'W).

*Specimen:* Single adult female: holotype, NMC (Ottawa) Cat. No. 1988-0035.

*Etymology:* The generic name alludes to the similarity between the new genus and *Lernaepodina* Wilson, 1915; the specific name after the host, *Synaphobranchus kaupi*.

**Description**

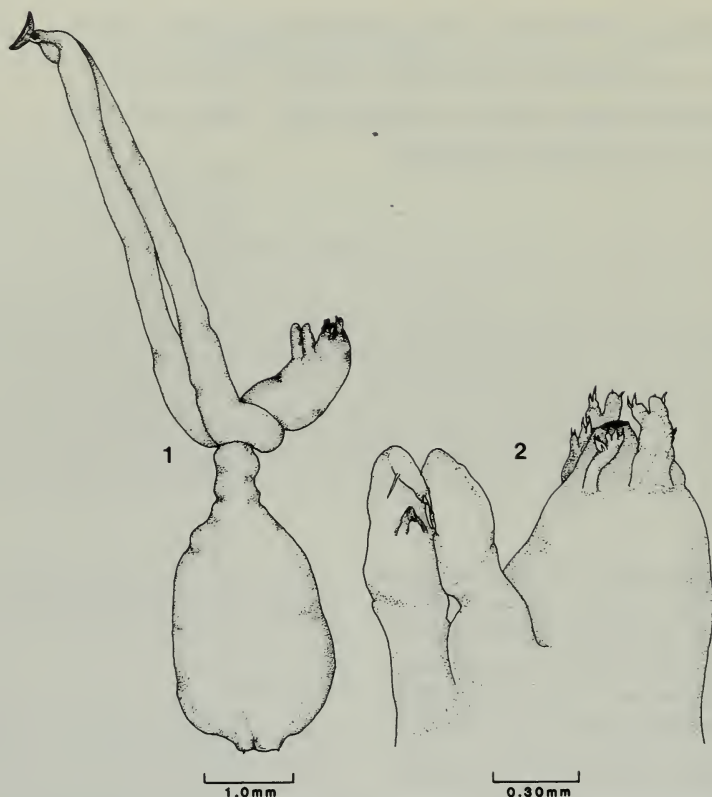
Lernaeopodidae: cephalothorax (Figs. 1, 2) subcylindrical, tapering anteriorly, 1/3 length of trunk and inclined dorsally to it. Dorsal shield absent or indiscernible. Trunk bulb-shaped, widest posteriorly, narrow anteriorly, with rounded posterolateral corners and convex lateral margins. Posterior margin rounded, provided with 2 reduced uropods at center. Oviduct orifices dorsal to anus, at center of posterior margin. Uropods (Fig. 7) bluntly truncate lobes, minute, armed at ends with 2 long, stout setae. Egg sacs missing.

First antenna (Fig. 3) 2-segmented; basal segment elongate, slightly expanded at base; terminal segment short, subcylindrical, armed at apex with 6 setae. Apical armature: setiform element (2), gibber with 2 elongate setae (complex 5), stout seta 4, short, tubercle 1 and spiniform seta 6. Whip and solus not found. Second antenna (Fig. 4) nonprehensile: sympod 2-segmented; exopod unsegmented, apex rounded, unspinulated, provided with 2 large and 1 slightly smaller papillae; endopod 2-segmented, terminal segment with 1 large degenerate hook and 1 attenuated process; ventral surfaces of penultimate and terminal segments spinulated. Mandible indistinct. First maxilla (Fig. 5) with lateral exopod carrying 2 short setae, dorsolateral margin unspinulated; single seta at base; endopod with 3 papillae, each provided with 1 seta. Second maxillae (Fig. 1) cylindrical, elongate, 1 1/2 length of trunk, fused at tips; bulla circular, plano-convex; manubrium small, slender.

Maxilliped (Fig. 6a) with large, elongate corpus; corpus provided with single large spiniform seta; myxal region with medial concavity and a rounded elevation armed with 1 small, blunt seta; concavity provided with several indistinct thornlike denticles; subchela 1/3 length of corpus, with 2 small apical setae (Fig. 6b); barb long, stout, 1/2 length of claw; claw large, slightly recurved, without accessory teeth.

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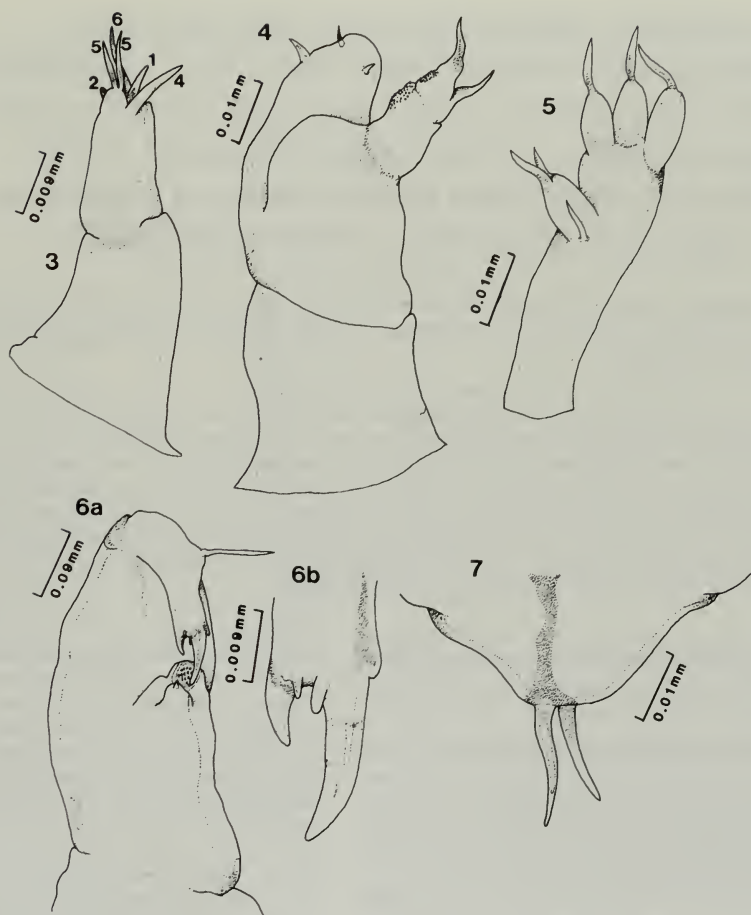
FIGURES 1, 2. 1. *Pseudolernaeopodina synaphobranchi* n. gen., n. sp., habitus, lateral. 2. Anterior end of cephalothorax, lateral (after clearing).

### DISCUSSION

As the generic name suggests, *Pseudolernaeopodina synaphobranchi* n. gen., n. sp. is more closely aligned to *Lernaeopodina* Wilson, 1915, than any other lernaeopodid genera. It possesses several features that are characteristic of *Lernaeopodina* such as: (1) elongate second maxillae with a simple bulla (although in *Lernaeopodina* the bulla can be bulbous as opposed to plano-convex as is found in *P. synaphobranchi*, (2) a trunk of similar shape and size, (3) a first maxilla with an exopod armed with 2 short setae, (4) a second antenna with the ventral surfaces of the endopod finely spinulated, and (5) the absence of a dorsal shield. *Pseudolernaeopodina synaphobranchi* is distinct from *Lernaeopodina* in the following: (1) in *Lernaeopodina* the cephalothorax is much more elongate than is characteristic of the new genus. (2) *Pseudolernaeopodina synaphobranchi* n. gen., n. sp. lacks any trace of posterior processes, having instead much reduced uropods. *Lernaeopodina* exhibits well-developed posterior processes of various size (de-

pending upon the species), all known species having posterior processes many times larger than the uropods found on *P. synaphobranchi*. (3) The apical armature of the endopod of the second antenna of all species of *Lernaeopodina* exhibits a large chitinous hook and at least 1 other large seta, and occasionally a small tubercle; *P. synaphobranchi* exhibits only 2 processes on the endopod of the second antenna, a feature not previously reported in the family Lernaeopodidae.

*Pseudolernaeopodina synaphobranchi* superficially resembles 2 other genera, *Pseudocharopinus* Kabata, 1964, and *Clavellistes* Shiino, 1963. *Pseudocharopinus*, a parasite of the spiracles and gill arches of elasmobranch fishes on both sides of the Atlantic, possesses some characters that are common both to it and the new species. These include a cephalothorax with dorsal flexure and a first maxilla of similar structure: endopod with 3 papillae and exopod with apical armature of 2 setae. *Pseudocharopinus* does, however, exhibit a distinct dorsal shield, short second maxillae, and large, well-developed posterior processes, all of which are lacking in *P. synaphobranchi*. *Clav-*



FIGURES 3-7. 3. First antenna, ventral. 4. Second antenna, ventral. 5. First maxilla, lateral. 6a. Maxilliped, lateral. 6b. Same, detail of subchela, ventral. 7. Uropod, lateral.

*ellistes* is a parasite of the moonfish, *Lampris*. It also possesses some features that are found in *P. synaphobranchi* such as a first antenna with similar apical armature (see Kabata, 1979: description of *C. lampri*), elongate second maxillae, and a cephalothorax with slight dorsal flexure. *Clavellistes*, like *P. synaphobranchi*, is also parasitic on the eye of its host. *Clavellistes* can be distinguished easily from the new species by the presence of a strong dorsal shield, a first maxilla with only 2 papillae on the endopod, a shorter, flatter cephalothorax, and, in particular, by the presence of a single large genital segment with a bifid tip. *Pseudolernaepodina synaphobranchi* may be tentatively aligned with the *Charopinus*-branch of the lernaepodid family tree (see Kabata, 1979). It is more closely related, as shown by its morphological features, to both the primitive ge-

nus *Lernaepodina* and the more advanced *Pseudocharopinus*, both members of this branch, possibly lying somewhere between these genera in evolutionary development.

Examination of additional specimens of *P. synaphobranchi* (when found) may reveal the dental formula of the mandible. This will further define the intrafamilial position of the new genus.

#### ACKNOWLEDGMENTS

I thank Dr. Ken Sulak for help in finding parasites during Oceanus cruise 183, Dr. Z. Kabata for reviewing this paper, and Kimberly Miller and Carla Carney for preparing the typescript.

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# SOME PARASITIC HELMINTHS FROM THE AMERICAN OYSTERCATCHER, *HAEMATOPUS PALLIATUS* TEMMINCK, FROM THE TEXAS GULF COAST AND THE COMMON PIED OYSTERCATCHER, *H. OSTRALEGUS* LINNAEUS, FROM NEW ZEALAND, INCLUDING *DILDOTAENIA LATOVARIVM* N. GEN. AND N. SP. (CESTODA: HYMENOLEPIDIDAE)

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**ABSTRACT:** *Acanthoparyphium spinulosum* (Digenea) and *Dildotaenia latovarium* n. gen. and n. sp. (Eucestoda) were recovered from an American oystercatcher, *Haematopus palliatus*, Galveston, Texas. This is the first report of *A. spinulosum* from the American oystercatcher. *Dildotaenia latovarium* was also recovered from common pied oystercatcher, *H. ostralegus*, from New Zealand. The new genus can be distinguished from all other hymenolepid genera that have a copulatory stylet in lacking a cirrus. The type species is characterized by the exaggerated width of its ovary. Of the other species of hymenolepids with 8 rostellar hooks from birds, *Dildotaenia latovarium* most closely resembles *Retinometra venusta*, but *R. venusta* has an armed cirrus and its testes are arranged in a line rather than in a triangle as in *D. latovarium*.

The American oystercatcher, *Haematopus palliatus* Temminck, ranges from the middle east coast of North America south to Chile and Argentina. It is a resident along the Texas gulf coast but occurs in relatively small numbers. The common pied oystercatcher, *H. ostralegus* Linnaeus, is found throughout most of the Old World, as well as the South Pacific.

The helminth fauna of members of the genus *Haematopus* is poorly known. Of the 8 extant species of oystercatcher there are reports of parasitic helminths from only *H. ostralegus* and *H. bachmani* Audubon (Table I). Most reports of helminths from oystercatchers have been from the common pied oystercatcher, *Haematopus ostralegus* from Europe and Russia. The purpose of this study is to provide additional information on the helminth fauna of oystercatchers.

## MATERIALS AND METHODS

Trematodes and cestodes were recovered from a moribund male *H. palliatus* found in the Galveston, Texas area on 5 June 1979. Cestodes of the same species were also found in *H. ostralegus* from Avon-Heathcote Estuary and the mouth of the Ashley River, New Zealand in 1979 and 1980. Specimens were fixed in AFA

under slight coverslip pressure, stained in Semichon's carmine, and mounted in Permount or Canada balsam. Some specimens were histologically sectioned by conventional paraffin technique. Figures were drawn with the aid of a camera lucida. Measurements are from whole mounts of adults and are given in  $\mu\text{m}$ , the mean followed by the range in parentheses, unless otherwise indicated.

## RESULTS

An American oystercatcher from Texas harbored *Acanthoparyphium spinulosum* Johnston, 1917 (Digenea: Echinostomatidae), and an undescribed species of hymenolepidid cestode, representing a new genus. The common pied oystercatchers from New Zealand were infected with the same species of tapeworm as found in the American oystercatcher from Texas.

## DESCRIPTION

### *Dildotaenia* n. gen. (Figs. 1-7)

**Diagnosis:** Hymenolepididae, Hymenolepidinae. Dorsoventrally flattened, becoming gradually wider toward gravid segments. All segments wider than long, craspedote. Scolex (Figs. 1-3) with retractable rostellum bearing 8 nitidoid (Fig. 4) hooks. Suckers simple, unarmed. Neck present, narrower than scolex. One set of protandrous male and female reproductive systems per segment. Genital pores unilateral. Three testes in triangle, 1 poral, 2 aporal (Figs. 5, 6). Genital ducts pass between osmoregulatory canals. Cirrus pouch oval, medial to osmoregulatory canals, containing small seminal vesicle. Cirrus absent. Long convoluted stylet originates within cirrus pouch and extends through a very thin sheath to genital atrium where it emerges

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TABLE I. *Cestodes and trematodes previously reported from members of the genus Haematopus.*

<i>Haematopus bachmani</i> (black oystercatcher)	
Cestode:	
1. <i>Hymenolepis alaskensis</i>	Deblock and Rausch, 1967
Trematode:	
1. <i>Maritrema gratosum</i>	Deblock and Rausch, 1972
<i>Haematopus ostralegus</i> (common pied oystercatcher)	
Cestodes:	
1. <i>Fimbriaria fasciolaris</i>	Jogis, 1963; Belopol'skaia, 1971
2. <i>Haploparaxis crassirostris</i>	van den Broek and Jensen, 1964
3. <i>Hymenolepis abortiva</i>	Jogis, 1963
4. <i>Hymenolepis aploparakoides</i>	Deblock, 1964
5. <i>Hymenolepis clandestina</i>	Threlfall, 1963
6. <i>Hymenolepis rectacantha</i>	Jogis, 1963; Threlfall, 1963; Deblock and Tran Van Ky, 1965
7. <i>Tschertkovilepis brachycephala</i>	Creplin, 1829
Trematodes:	
1. <i>Gymnophalloides oedemia</i>	James, 1964
2. <i>Gymnophallus choledochus</i>	Loos-Frank, 1968
3. <i>Himasthla leptosoma</i>	Threlfall, 1963
4. <i>Microphallus claviformis</i>	Deblock and Tran Van Ky, 1966
5. <i>Microphallus similis</i>	Deblock and Tran Van Ky, 1966
6. <i>Paramonostomum chabaudi</i>	van Strydonck, 1965a
7. <i>Parvatrema affine</i>	James, 1964; Belopol'skaia, 1966
8. <i>Parvatrema homoeotecnium</i>	James, 1964
9. <i>Psilostomum brevicolle</i>	Threlfall, 1963; van Strydonck, 1965b; Belopol'skaia, 1966; Loos-Frank, 1968

through a small unarmed, nipple-like projection of the atrium wall (Fig. 7). Accessory sacs absent. External seminal vesicle present. Ovary median (Fig. 6), much wider than long. Vitellarium compact, postovarian. Vagina opens into genital atrium posterior to stylet pore. Seminal receptacle present, persistent. Uterus appears as bilobed sac, becoming irregular sac when filled with eggs. Parasites of Charadriiformes.

#### Taxonomic summary

*Type species:* *Dildotaenia latovarum* n. sp.

*Etymology:* Genus named after "dildo" (from Italian, dilletto), referring to the surrogate cirrus in the genital atrium.

#### *Dildotaenia latovarum* n. sp.

(Figs. 1–7)

*Description* (based on 10 adult specimens): With characteristics of the genus. Strobila craspedote, apolytic, 10,000 (6,600–15,000) long, composed of 95–160 proglottids. Scolex 170 (150–195) wide by 309 (262–338) long. Suckers well developed, 88 (78–102) wide. Everted rostellum 168 (135–188) long with 8 hooks, each 56 (53–58) long, arranged in a circle. Immature proglottids wider than long, mature proglottids 810 (465–1,356) wide by 164 (97–233) long. Ovary 461 (385–670) wide by 44 (32–60) long. Vitellarium 128 (100–165) wide by 59 (48–72) long. Cirrus sac ovoid, transverse, 71 (61–86) wide by 123 (92–138) long. External seminal vesicle 53 (36–78) wide by 304 (244–500) long. Fully developed eggs not observed.

#### Taxonomic summary

*Type host:* *Haematopus palliatus*.

*Site of infection:* Small intestine.

*Locality:* Galveston, Texas; South Island, New Zealand.

*Holotype:* USNM Helm. Coll. No. 80271.

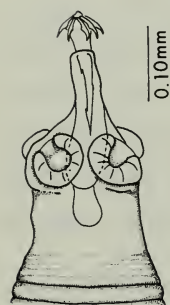
*Paratypes:* USNM Helm. Coll. Nos. 80272 and 80273; Texas A&M Cooperative Wildlife Regional Coll. No. 78A-38, Department of Wildlife and Fisheries Sciences, Texas A&M University.

*Etymology:* The species name refers to its large, laterally elongated ovary.

#### DISCUSSION

*Dildotaenia* n. gen. differs from all other hymenolepidid genera that have copulatory stylets in lacking a cirrus. Serial sections of the genital atrium clearly show the nipple-like projection in the genital atrium to be continuous with the atrial wall, not in any way attached to or extending from the cirrus pouch. As this structure serves as a cirrus substitute, we propose to call it the dildo. The new species superficially resembles members of the genus *Retinometra*. The only species of *Retinometra* so far described from charadriiform birds is *R. deblocki* (Schmidt and Neiland, 1968) Schmidt, 1986. It differs from *D. latovarum* in having a rostellum armed with 10 hooks that are 100–102 long. *Dildotaenia latovarum* can be distinguished from other species of hymenolepids with 8 rostellar hooks by the exaggerated width of its ovary (385–675). Of described species of hymenolepids with 8 rostellar





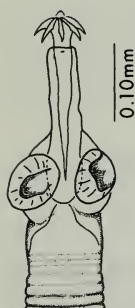
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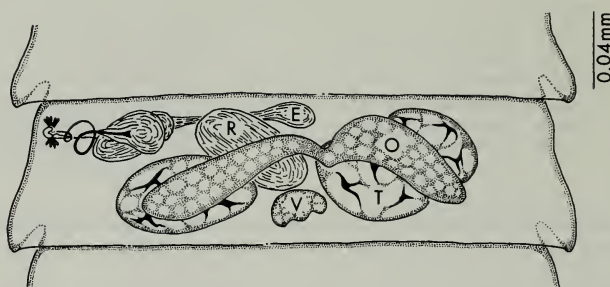
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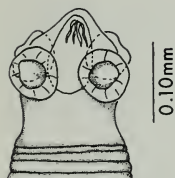
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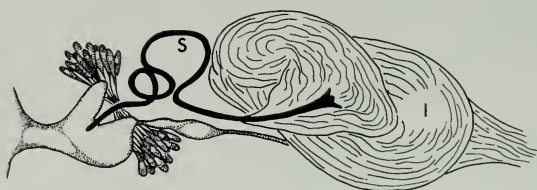
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FIGURES 1-7. *Dildotaenia latovarum* n. gen. and n. sp. from *Haematopus palliatus* and *H. ostralegus*. 1. Scolex partially retracted. 2. Scolex fully extended. 3. Scolex fully retracted. 4. Hook from rostellum. 5. Immature proglottid. 6. Mature proglottid. E = external seminal vesicle, R = seminal receptacle, O = ovary, T = testis, V = vitellarium. 7. Enlarged view of the genital atrium and internal seminal vesicle regions. S = copulatory spicule, I = internal seminal vesicle.

hooks, *D. latovarum* most closely resembles *Retinometra venusta* (Rosseter, 1897) Railliet and Henry, 1909, which is unlike *D. latovarum* in the following characters: it has an armed cirrus,

the blade of its rostellar hooks is longer than the base, and its testes are arranged in a line rather than in a triangle.

*Acanthoparyphium spinulosum* has a cosmo-

politan distribution and was originally described from a plover in Sydney, Australia (Johnston, 1917). It has also been reported from black-bellied plover, *Squatarola squatarola*, in Japan (Yamaguti, 1934), and in the United States adults have been reported from black-bellied plover and American avocet, *Recurvirostra americana*, in California (Martin and Adams, 1961). Metacercariae of *A. spinulosum* have been reported from oyster, *Crassostrea virginica*, along the Texas gulf coast by Little et al. (1966). The present study is the first report of *A. spinulosum* from American oystercatcher.

#### ACKNOWLEDGMENT

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# **CLOACOTAENIELLA TRINGAE N. GEN., N. SP. (CESTOIDEA: HYMENOLEPIDIDAE) FROM A REDSHANKS, TRINGA TOTANUS (CHARADRIIFORMES) IN ISRAEL**

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**ABSTRACT:** *Cloacotaeniella* n. gen. is similar to *Cloacotaenia* Wolffhügel, 1938, but differs in possessing a spinous accessory sac attached to the vagina, in lacking a male gonopore and an apical organ on the scolex, and in having an extensive, irregular uterus. The type species, *C. tringae* n. sp. was found in a charadriiform bird, *Tringa totanus*, in Israel.

In a previous publication (Schmidt et al., 1986) survey data on cestodes from Israel and Sinai were presented. In that paper *Cloacotaenia* sp. was reported from *Tringa totanus*, with the comment that it was to be discussed further in a later publication. The present report describes that species, for which a new genus is proposed. The single specimen was fixed in 5% formalin, stained with hematoxylin, and mounted in balsam by conventional technique. Measurements are in  $\mu\text{m}$  unless otherwise indicated.

## ***Cloacotaeniella* n. gen.**

**Diagnosis:** Hymenolepididae, Hymenolepidinae. Dorsoventrally flattened, becoming wider evenly throughout length of strobila. All segments wider than long, craspedote, with long velum. Scolex massive, with 4 simple suckers and no apical organ. Slender neck present. Osmoregulatory canals simple. One set of reproductive organs per segment. Genital pores unilateral, dextral. Genital atrium absent. Genital ducts pass between osmoregulatory canals. Testes 3, posterior to ovary, between outer limits of osmoregulatory canals. Cirrus pouch narrow, nearly reaching midline of proglottid. Cirrus armed. External and internal seminal vesicles present. Male genital pore absent. Cirrus opens into spinous accessory sac which is a diverticulum of the vagina. Ovary median, irregular, transversely elongate. Vitellarium posterior to ovary. Vagina dorsal or anterior to cirrus pouch, opening alone on anterior half of proglottid margin. Seminal receptacle large. Uterus develops as a thin-walled, transverse sac that when gravid becomes irregular, filling proglottid almost to lateral margins. Oncospheres each apparently surrounded by 2 thin membranes.

**Type (and only) species:** *Cloacotaeniella tringae* n. sp.

**Etymology:** Reflecting its superficial resemblance to *Cloacotaenia*.

## **Remarks**

*Cloacotaeniella* resembles *Cloacotaenia* Wolffhügel, 1938 (syn. *Orlovilepis* Spasskii et Spasskaja, 1954), to which it may be closely related. *Cloacotaenia* is also monotypic, consisting of the species *C. megalops* (Nitzsch in Creplin, 1829) Wolffhügel, 1938. The species is common worldwide in anseriform birds, but has not been reported from a charadriiform bird. *Cloacotaeniella* differs from *Cloacotaenia* in 3 easily distinguished characteristics: (1) its vagina gives rise to a spiny accessory sac into which the cirrus opens, whereas no such sac exists in *Cloacotaenia*; (2) the uterus of *Cloacotaeniella* forms as and remains an irregular sac that when gravid nearly reaches the lateral margins of the proglottid, whereas that of *Cloacotaenia* is formed as and remains a thick-walled, oval chamber in the center of the proglottid; (3) *Cloacotaeniella* lacks an apical organ.

## ***Cloacotaeniella tringae* n. sp. (Figs. 1-6)**

## **Description**

Strobila about 40 mm long, greatest width 1.04 mm at posterior end. Scolex (Fig. 1) 1.15 mm wide (length cannot be determined due to position on slide). Suckers round, simple, unarmed 190-200 wide. Neck about 600 long. Genital primordia appear in first visible segment.

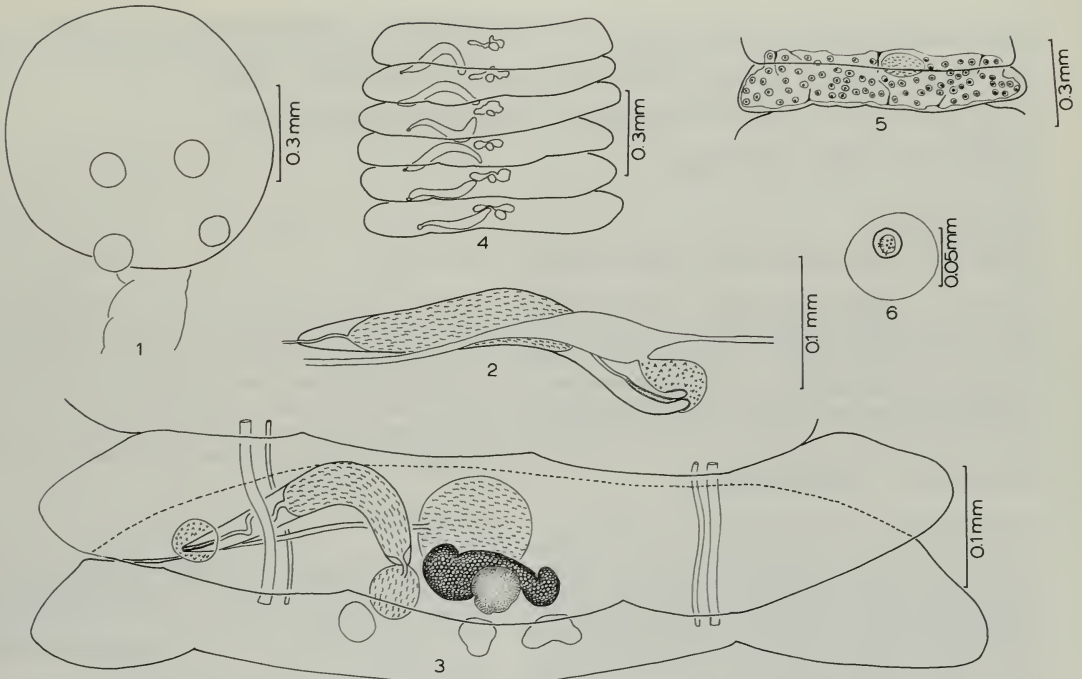
Proglottids craspedote; velum long, with irregular margin. Mature proglottid ( $n = 4$ ) 910-930 wide, 275-285 long. Gravid proglottid ( $n = 4$ ) 0.900-1.04 mm wide, 240-250 long. Ventral osmoregulatory canals about 8 wide, joined near rear margin of proglottid by simple, transverse anastomosis. Dorsal canals simple, about 4 wide. Genital atrium absent. Genital pores unilateral, near anterior margin of proglottid. Genital ducts (Fig. 2) pass between osmoregulatory canals. Reproductive systems (Fig. 3) mature at about same time.

**Male genitalia:** Three testes (some proglottids with 1 or 2) irregularly arranged behind ovary; each 30-40 wide ( $n = 12$ ). External seminal vesicle 80-90 greatest width ( $n = 6$ ). Cirrus pouch narrow, reaching midline of proglottid, straight or variously curved (Fig. 4); 250-280 long, 30-40 maximum width ( $n = 10$ ). Internal seminal vesicle 130-160 long, 30-38 wide ( $n = 10$ ). Cirrus 40-50 long, 8-10 wide ( $n = 5$ ) covered with tiny spines. Cirrus opens into spinous accessory sac (Fig. 2).

**Female genitalia:** Ovary median, irregular, trans-

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FIGURES 1–6. *Cloacotaeniella tringae* n. sp. from *Tringa totanus* in Israel. 1. Scolex, tilted toward the viewer. 2. Terminal genital ducts, showing dorsal vagina. 3. Mature proglottid, ventral view, testes somewhat atrophied. 4. Young mature proglottids, showing variations in shapes of cirrus pouches. 5. Gravid proglottid. 6. Egg.

versely elongate, 60–100 long, 140–200 wide ( $n = 5$ ). Vitellarium compact, postovarian 40–60 long, 60–80 wide ( $n = 5$ ). Vagina dorsal or anterior to cirrus pouch, opening independently on margin of proglottid. About 100 from distal end of vagina, accessory sac lined with small v-shaped spines, protrudes ventroposteriad from it (Fig. 2). The cirrus opens into this sac. Seminal receptacle ovoid, 140–220 greatest length ( $n = 6$ ). Uterus (Fig. 5) forms as simple, transverse sac, becoming irregular and filling most of proglottid into extreme lateral margins when gravid. Eggs (Fig. 6) ( $n = 20$ ) with outer membrane 40–50 wide; middle membrane 34–40 wide. Oncosphere 16–22 wide; hooks about 10 long.

*Type host:* Redshanks, *Tringa totanus* (Linnaeus, 1758) (Charadriiformes).

*Type locality:* Kefar Messaryk, Israel.

*Habitat:* Large intestine.

*Holotype specimen:* Hebrew University, Jerusalem, Parasitological Collection, holotype no. 870.

*Etymology:* Named after the genus of its host.

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# PHYLOGENETIC ANALYSIS OF THE NORTH AMERICAN SPECIES OF THE GENUS *HAPALORHYNCHUS* STUNKARD, 1922 (TREMATODA: SPIRORCHIIDAE), BLOOD-FLUKES OF FRESHWATER TURTLES

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**ABSTRACT:** Cladistic analysis of the North American species of the blood-fluke genus *Hapalorhynchus* Stunkard, 1922, was completed. *Hapalorhynchus gracilis*, *H. brooksi*, *H. stunkardi*, *H. reelfooti*, and *H. foliorchis* form a monophyletic group based on the presence of an enlarged pars prostatica. *Hapalorhynchus brooksi* has retained a cirrus sac (plesiomorphy), whereas the remaining species show a secondary loss of this structure. *Hapalorhynchus stunkardi* and *H. reelfooti* are considered sister-species based on the anterior distribution of the vitellaria. *Hapalorhynchus foliorchis* and *H. gracilis* are sister-species, but may represent age-related variants of a single entity. The presence of *H. reelfooti* and *H. stunkardi* in *Sternotherus* appears to represent colonization from the snapping turtle, *Chelydra serpentina*.

Stunkard (1922) erected the genus *Hapalorhynchus* to accommodate blood-flukes from the snapping turtle, *Chelydra serpentina*, that lacked a cirrus sac and possessed an acetabulum and a large tricornuate egg. Since that time, 6 additional species of this genus have been described from freshwater turtles in North America (see Platt, 1988, for a review). The purpose of this study is to examine the phylogenetic relationships of the North American species of *Hapalorhynchus* using cladistic analysis.

## MATERIALS AND METHODS

The specimens that formed the basis of this analysis are described elsewhere (Platt, 1988). *Hapalorhynchus evaginatus* Byrd, 1939, was not included in this analysis as Byrd (1939) did not describe the reproductive system, and I could not see it in the holotype (pers. obs.).

Cladograms were constructed using PAUP, a computer-based method for inferring phylogenies using parsimony criteria (Swofford, 1985). In addition, alternative cladograms were evaluated, character-state distributions analyzed, and consistency indices calculated using MacClade (Maddison, 1986).

Character state polarity was determined by the out-group method (Wiley, 1981). Species of the related genus *Coelotremata* Mehra, 1933, were used as the out-group. *Coelotremata*, although considered by some authors to be synonymous with *Hapalorhynchus*, possesses several attributes that make it ideal as the out-group. The position of the genital pore (opening dorsally on the left) is unique to these genera among the Spirorchiidae, indicating a high probability that they are a monophyletic group. In addition, no species of *Coelotremata* have been reported from North America. Watrous and Wheeler (1981) indicated that it is not

necessary for a functional out-group to be a taxonomic out-group as well. Therefore, the possible synonymy of these genera is not relevant to the following analysis. The use of *Amphiorchis* Price, 1934, as the out-group did not affect the form of the cladogram.

## RESULTS

### Characters

Each character used in the analysis is listed below; the character states are polarized by comparison with the out-group. The plesiomorphic condition is coded as 0; derived conditions are identified as 1 or 2. Missing data are coded as ?.

- 1) Sucker ratio: width of the ventral sucker divided by the oral sucker width: 2 states; 0 =  $>1$ , 1 =  $<1$ .
- 2) Esophageal diverticula: outpocketings of the esophagus: 2 states; 0 = absent, 1 = present.
- 3) Cirrus sac: a membrane surrounding the distal ejaculatory duct and prostatic complex: 2 states; 0 = present, 1 = absent.
- 4) Shape of the ovary: 2 states; 0 = pyriform, 1 = oval.
- 5) Shape of the stem of the excretory vesicle: 2 states; 0 = straight to slightly sinuous, 1 = branched.
- 6) Anterior distribution of vitellaria: 2 states; 0 = stop at cecal bifurcation, 1 = extend to mid-esophagus.
- 7) Postcecal distance: distance from the termination of the ceca to the posterior end of the worm divided by total length and expressed as a percentage: 2 states; 0 =  $<20\%$ , 1 =  $>20\%$ .
- 8) Cecal diverticula: outpocketings of the an-

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TABLE I. Data matrix of character states for the North American species of *Hapalorhynchus* Stunkard, 1922.

Species	Character									
	1	2	3	4	5	6	7	8	9	10
<i>Coeuritrema</i> Mehra, 1933	0	0	0	0	0	0	0	0	0	0
<i>H. gracilis</i> Stunkard, 1922	0	0	1	0	0	0	1	0	1	2
<i>H. stunkardi</i> Byrd, 1939	0	0	1	0	0	1	0	0	1	1
<i>H. reelfooti</i> Byrd, 1939	0	0	?	0	0	1	0	0	1	?
<i>H. foliorchis</i> Brooks and Mayes, 1975	0	0	1	1	0	0	1	0	1	2
<i>H. albertoi</i> Lamothe-Argumedo, 1978	1	1	0	0	0	0	0	0	0	?
<i>H. brooksi</i> Platt, 1988	0	0	0	0	1	0	0	1	1	0

terolateral portion of the intestinal ceca: 2 states; 0 = absent, 1 = present.

9) Pars prostatica: 2 states; 0 = not enlarged, 1 = conspicuous.

10) Egg shape: 3 states; 0 = bipolar, 1 = oval, 2 = tricornuate.

#### Parasite phylogeny

The distribution of character states is provided in Table I. Presence or absence of a cirrus sac could not be determined for *H. reelfooti* Byrd, 1939. That area is obscured on the type specimen (pers. obs.), and the shape of the egg is unknown for both *H. reelfooti* and *H. albertoi* Lamothe-Argumedo, 1978.

The cladogram (Fig. 1) has a consistency index of 1, indicating an absence of homoplasy in the characters examined. However, *H. albertoi* cannot be included with the remaining North American species that are recognized as a monophyletic group, based on the presence of an enlarged pars prostatica. *Hapalorhynchus brooksi* retains a cirrus sac, a plesiomorphy, whereas in the remaining 4 species the prostatic cells are diffuse and somewhat irregularly arranged around the pars prostatica and distal ejaculatory duct. Egg shape (Fig. 2) shows 2 changes from the plesiomorphic condition; oval in *H. stunkardi* and tricornuate in the *H. gracilis*–*H. brooksi* lineage.

#### DISCUSSION

The species of *Hapalorhynchus* reported from turtles in the United States (*H. brooksi* through *H. foliorchis*; Fig. 1) form a monophyletic group. The only ambiguity in the cladogram is the shape of the egg (Fig. 2). Species nominally assigned to *Coeuritrema* are reported as having eggs with bipolar filaments (Yamaguti, 1971), and this is considered to be the plesiomorphic condition. Three states exist within the U.S. fauna; bipolar filaments, filaments lacking, and tricornuate. Identification of the egg type for *H. reelfooti* should resolve the polarity of this character. If the egg of *H. reelfooti* is tricornuate, then char-

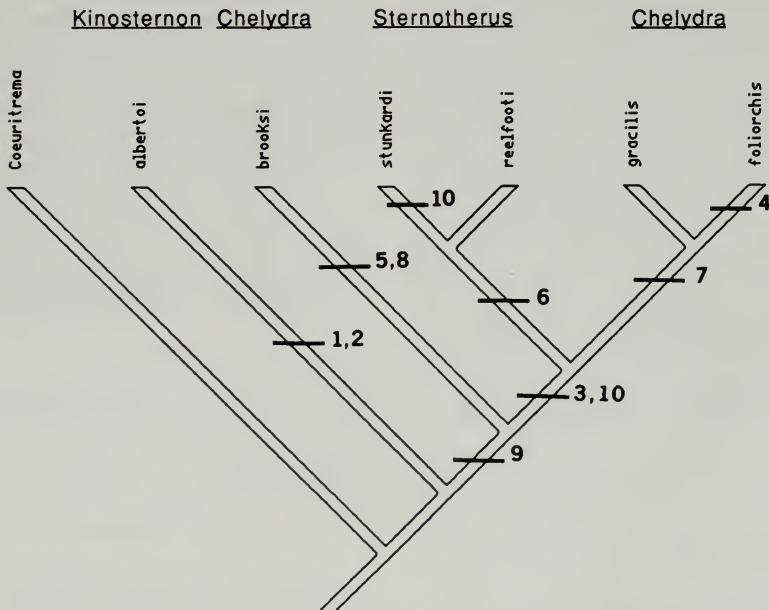


FIGURE 1. Cladogram of the North American species of *Hapalorhynchus* Stunkard, 1922, with host genera. Lines represent appearance of apomorphic character states (see text for details).



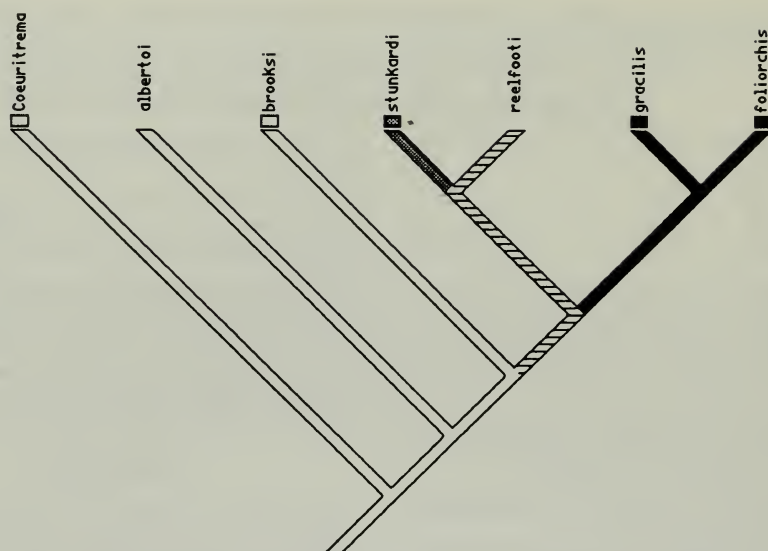


FIGURE 2. Cladogram of North American species of *Hapalorhynchus* Stunkard, 1922, showing the transformation of character 10, egg shape. Shaded areas represent an apomorphic condition; hatched area indicates equivocal information.

acter transformation would occur as shown in Figure 3a; however, if the egg is bipolar, then the transformation would occur as shown in Figure 3b.

Although these species have been reported infrequently in the literature, they appear to exhibit narrow co-accommodation with their respective hosts (Platt, 1988; see Fig. 1), as defined by Brooks (1979). Three species, *H. brooksi*, *H. foliorchis*, and *H. gracilis*, were described from the snapping turtle, *Chelydra serpentina*. The remaining species, *H. stunkardi* and *H. reelfooti*, were described from musk turtles, *Sternotherus carinatus* and *S. odoratus*, respectively (Byrd, 1939).

It is difficult to generalize about the geographic distribution of these species given the paucity of reports, but some observations are worth noting. *Hapalorhynchus brooksi* has been found in Virginia (Platt, 1988) and eastern Nebraska (Brooks and Mayes, 1976). Although these areas do not represent the eastern and western limits of the range of the snapping turtle (Carr, 1952), it is clear that *H. brooksi* has a wide distribution. In addition, *H. gracilis* has been reported from Virginia (Platt, 1988), Wisconsin (Guilford, 1959), and Indiana (Stunkard, 1922). It is quite possible that both species will be found throughout the range of *Chelydra serpentina*.

This analysis also calls into question the validity of *H. foliorchis*. *Hapalorhynchus gracilis* and *H. foliorchis* are separated on the basis of

the size and shape of the ovary, and the size of the testes. The former species exhibits the plesiomorphic condition for the ovary, i.e., pyriform. The ovary in *H. foliorchis* is oval, but located in the same position as the large lobe of *H. gracilis*. The type specimens of *H. foliorchis* are consistent with the original description (Brooks and Mayes, 1975); however, the extension of the lateral arm of the ovary and testis size appear somewhat variable in *H. gracilis* (pers. obs.) and may be a function of age. Further study may reveal that these taxa are conspecific, and both *H. gracilis* and *H. brooksi* would share a similar range. If *H. foliorchis* is valid, this would

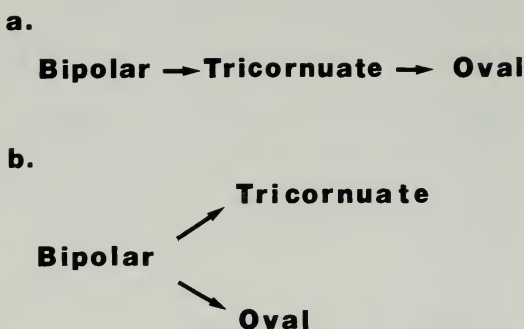


FIGURE 3. Possible transformations of character 10 (egg shape) from the ancestor of the *stunkardi-foliorchis* line (point a). a. Transformation series if the bipolar condition is plesiomorphic. b. Transformation series if the tricornuate condition is plesiomorphic.

represent a speciation event in the western portion of the host range.

The presence of the *H. stunkardi*–*H. reelfooti* lineage in *Sternotherus* raises some interesting questions regarding parasite transfer. Recent studies of turtle phylogeny (Gaffney, 1984) indicate that *Chelydra* and *Sternotherus* are not closely related; however, they are similar in both habit and habitat (Carr, 1952). The position of this lineage suggests a colonization event (Brooks, 1981) from the line established in *Chelydra*. It is worth noting that an alternative colonization hypothesis is possible. If the North American fauna originated in the Kinosternidae, as evidenced by the presence of *H. albertoi* in *Kinosternon leucostomum*, then *Hapalorhynchus* may have invaded the snapping turtle on 2 occasions: first by *H. brooksi* and subsequently by the ancestor of the *H. gracilis*–*H. foliorchis* line.

*Hapalorhynchus albertoi* is not included in the same monophyletic group as the other North American species of the genus. The absence of any synapomorphy linking *H. albertoi* with the remaining North American species suggests its inclusion in *Coeuritrema*, however, at the present time there are no recognized synapomorphies that establish *Coeuritrema* as a monophyletic taxon. This again raises the issue of the synonymy of these genera. Mehra (1933) erected *Coeuritrema* for 2 species of blood-flukes from Indian turtles, which bore a remarkable similarity to *Hapalorhynchus* except that they possessed a distinct cirrus and cirrus sac. North American students of this group (Price, 1934; Byrd, 1939; Brooks and Mayes, 1976) have considered the two genera synonymous, but Mehra (1933, 1939) and Yamaguti (1971) recognized them as distinct entities. These issues have recently been reviewed (Brooks and Mayes, 1976; Bourgat and Kulo, 1987; Platt, 1988) but not resolved. The possession of a cirrus sac and absence of an enlarged pars prostatica in *Coeuritrema* are symplesiomorphies and hence cannot be used to delineate a distinct taxonomic entity (Wiley, 1981). For the present, *Coeuritrema* should be recognized as a junior synonym of *Hapalorhynchus*. Examination of a range of specimens of *Coeuritrema* may result in the identification of synapomorphies that would establish it as a valid genus.

Strict adherence to the tenets of phylogenetic systematics requires a classification that precisely replicates the branch points in the cladogram (Hennig, 1966). At the present time I feel that

this action would cause unnecessary confusion and needlessly complicate the classification. Employing the sequencing convention (Wiley, 1981) would still require the erection of several subgenera to adequately recognize the nonsymmetrical branches in Figure 1. Due to the unsettled nature of the affinities of *Coeuritrema*, I prefer to recognize this entire assemblage as *Hapalorhynchus* s.l. until such time as the relationships of these genera are clarified.

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## EXPERIMENTAL *SARCOCYSTIS HOMINIS* INFECTION IN CATTLE: LESIONS AND ULTRASTRUCTURE OF SARCOCYSTS

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**ABSTRACT:** Five calves inoculated orally with  $10^5$ – $10^6$  sporocysts of *Sarcocystis hominis* from human feces were necropsied 10, 18, 24, 111, and 222 days postinoculation (DPI). Calves became febrile ( $>40.1$ – $41$  C) between 10 and 24 DPI and developed mild anemia (packed cell volumes were reduced by 40% of initial values) between 29 and 57 DPI but otherwise remained clinically normal. Focal hepatitis, mesenteric lymphadenitis, and myocarditis were seen in calves at 10, 18, and 24 DPI. No stages of the parasite were found at any of these times except for a few merozoites in macrophages associated with myocardial lesions in the calf necropsied 24 DPI. Mature sarcocysts at 111 and 222 DPI were up to  $950\text{ }\mu\text{m}$  long and their walls were up to  $6\text{ }\mu\text{m}$  thick. They were found only in skeletal muscles. One immature sarcocyst was seen in the myocardium of the calf at 222 DPI.

Cattle are intermediate hosts for 3 species of *Sarcocystis*: *S. cruzi*, *S. hirsuta*, and *S. hominis*. Of these, *S. cruzi* is highly pathogenic and *S. hirsuta* is mildly pathogenic, but little is known of the pathogenicity or biology of *S. hominis* in cattle (Gestrich et al., 1975a; Mehlhorn et al., 1975a; Fayer and Dubey, 1986). We report the distribution of parasites and lesions as well as ultrastructure of the sarcocysts of *S. hominis* in experimentally infected cattle.

### MATERIALS AND METHODS

*Sarcocystis hominis* sporocysts were obtained from the feces of a human volunteer in the Federal Republic of Germany (FRG) who ingested naturally infected beef in Turkey. Sporocysts were cleaned, stored in antibiotics, and shipped by air from FRG to the Animal Parasitology Institute (API). The sporocysts had been stored at 5 C for up to 12 mo before 5 calves in 2 experiments were inoculated orally at the API.

In experiment 1, 1 3-wk-old calf (calf 1) received  $10^6$  sporocysts and another of the same age (calf 2)  $10^5$ . Calves 1 and 2 were euthanized and necropsied 111 and 222 DPI, respectively.

In experiment 2, 3 2-mo-old calves received  $10^6$  (calves 3, 4) or  $2.5 \times 10^5$  (calf 5) *S. hominis* sporocysts and were euthanized and necropsied 13, 18, and 24 DPI, respectively. Calf 6 was an uninoculated control of the same age that was euthanized and necropsied on the same day as calf 5.

Inoculated calves were housed separately from the uninoculated calf for 7 days after inoculation; all waste and bedding was bagged in plastic within the pen and then incinerated to kill sporocysts that might have passed unexcysted in their feces. Calves were fed milk replacer until 6–8 wk of age and were then fed grain and hay. They were housed indoors in individual pens with straw as bedding.

Temperatures and hematological data were obtained from the calves in experiment 1. Temperatures were recorded daily from the day of inoculation until necropsy. Blood was collected weekly (in tubes containing sodium ethylenediaminetetraacetic acid) from each calf during the same time period. Packed cell volumes (PCV) were determined using a microhematocrit.

The calves were euthanized and then immediately necropsied. In experiment 1, portions of eyes, pituitary, salivary and adrenal glands, thymus, lungs, heart, diaphragm, spleen, kidneys, liver, gall bladder, urinary bladder, omentum, rumen, reticulum, omasum, abomasum, small and large intestines, esophagus, skeletal muscle from the thigh, lymph nodes (superficial cervical, mandibular, retropharyngeal, mediastinal, hepatic, gastric, mesenteric, subiliac), cerebrum, cerebellum, pons, medulla, spinal cord, tongue, and skin were fixed in 10% neutral buffered formalin (NBF). In experiment 2, sections of small and large intestines were taken at 0.3-m intervals and associated lymph nodes of each calf were fixed in NBF. Paraffin-embedded sections were cut at  $6\text{ }\mu\text{m}$  and stained with hematoxylin and eosin (HE).

Esophageal muscle from calf 1 was fixed in 1% glutaraldehyde and 4% formaldehyde mixture and processed for transmission electron microscopy (TEM) as described by Speer and Dubey (1982). Skeletal muscles from calf 2 fixed in NBF were processed for TEM. Specimens were examined with a JEOL 100 CX electron microscope.

### RESULTS

Calves 1 and 2 became febrile ( $>40.1$ – $41$  C) but otherwise remained clinically normal. Calf 1 was febrile on 9, 15–18, and 24 DPI and calf 2 was febrile on 14 and 16–21 DPI. Both calves became mildly anemic; their PCV's were reduced from an initial percentage of 40–23, 22, 29, 28, and 35 on 29, 36, 43, 50, and 57 DPI, respectively, for calf 1, and 29, 25, 39 on 22, 29, and 36 DPI, respectively, for calf 2.

Except for hyperemia and enlargement of the

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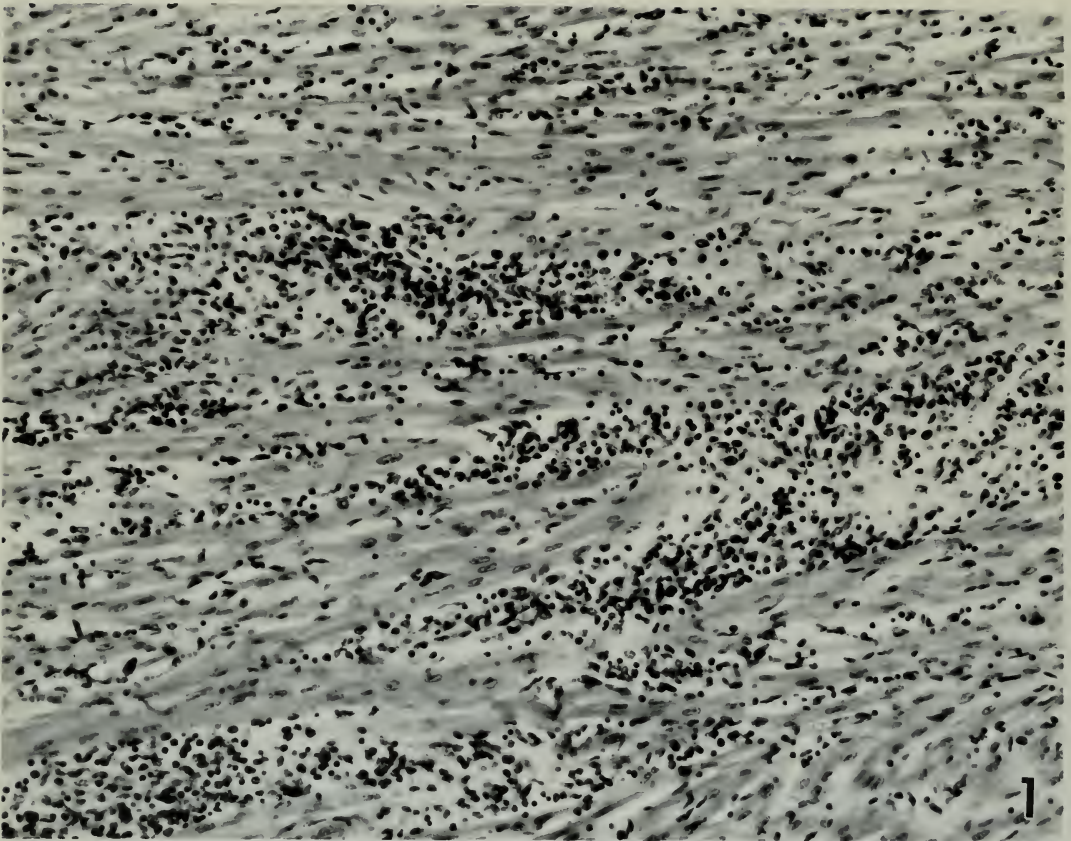


FIGURE 1. Severe mononuclear cell infiltration in the myocardium of calf 5, 24 DAI. H&E stain.  $\times 175$ .

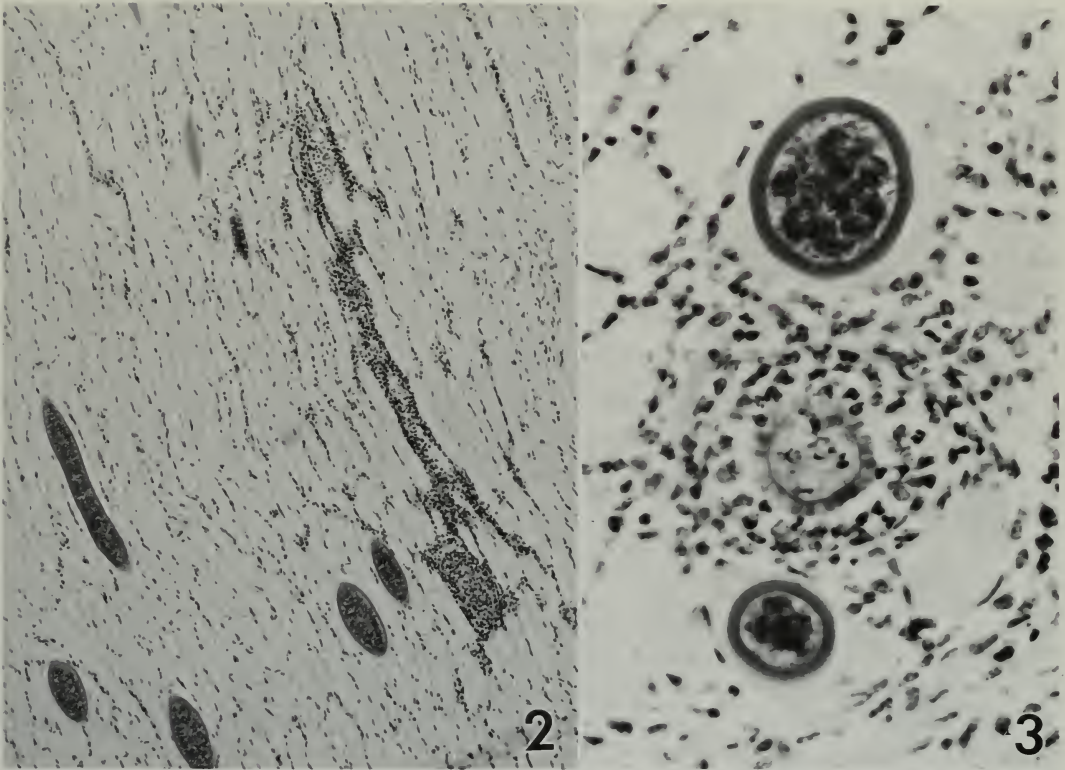
mesenteric lymph nodes, in calves 3–5, no macroscopic lesions were seen.

Neither schizonts nor sarcocysts of *Sarcocystis* were seen in the tissues of calves 3–6. However, microscopic lesions were present in the liver, heart, and gut-associated lymph nodes in calves 3–5. Hepatitis was characterized by mononuclear cell infiltrations around the portal triads and small bile ducts. Lymphadenitis was due to hyperplasia of reticuloendothelial cells; in calf 5, there was also hemosiderosis. Myocarditis was characterized by edema and mononuclear cell infiltration (primarily lymphocytes and macrophages) in the myocardium (Fig. 1). Myocarditis was most severe in calf 5 and involved the epicardium, endocardium, both atria, and both ventricles. A few individual merozoites were found within macrophages in the heart of calf 5.

Sarcocysts were found in histologic sections of the skeletal muscles of calf 1, particularly in the esophagus (Fig. 2). They were up to  $750\ \mu\text{m}$  long and  $80\ \mu\text{m}$  wide and had walls  $5\text{--}6\ \mu\text{m}$  thick. Most sarcocysts contained only bradyzoites. A

few sarcocysts were degenerating (Fig. 3) and were surrounded by neutrophils and mononuclear cells. Sarcocysts or lesions were not seen in any other tissue including the heart. In calf 2, there were fewer sarcocysts than in calf 1, but they were found in the same locations as in calf 1. One immature sarcocyst ( $77 \times 33\ \mu\text{m}$ ) containing only merozoites was found in the heart. The sarcocysts were up to  $950\ \mu\text{m}$  long and  $120\ \mu\text{m}$  wide, but the sarcocyst wall thickness was the same as in calf 1. Several sarcocysts were degenerating and surrounded by lymphocytes.

Ultrastructurally, the primary sarcocyst wall (pcw) of *S. hominis* consisted of a parasitophorous vacuole membrane (pvm) and an electron-dense layer  $50\ \text{nm}$  thick ( $43\text{--}62.5\ \text{nm}$ ;  $n = 20$ ) immediately beneath the pvm (Figs. 4, 5). The pcw was folded into villar projections that measured  $5.7 \times 0.6\ \mu\text{m}$  ( $5.3\text{--}6 \times 0.4\text{--}0.9\ \mu\text{m}$ ;  $n = 10$ ) at 111 DPI and  $7 \times 1.4\ \mu\text{m}$  ( $6.5\text{--}7.5 \times 1.1\text{--}1.8\ \mu\text{m}$ ;  $n = 10$ ) at 222 DPI. The cores of the villar projections of cysts of both ages were similar ultrastructurally, containing widely scattered



FIGURES 2, 3. Sections of diaphragm of calf 111 DAI with *Sarcocystis hominis* sporocysts. H&E stain. 2. Mononuclear cell infiltrations and 7 sarcocysts.  $\times 75$ . 3. A degenerating sarcocyst infiltrated with neutrophils and 2 sarcocysts without host cells.  $\times 300$ .

granules and long filaments that extended from the base to the tip of the projection, where they were embedded in the electron-dense layer of the pcw. At 222 DPI the core filaments were arranged in loosely associated bundles and some of the granules were larger and more electron-dense than those at 111 DPI. In contrast, those at 111 DPI had small granules and uniformly arranged filaments.

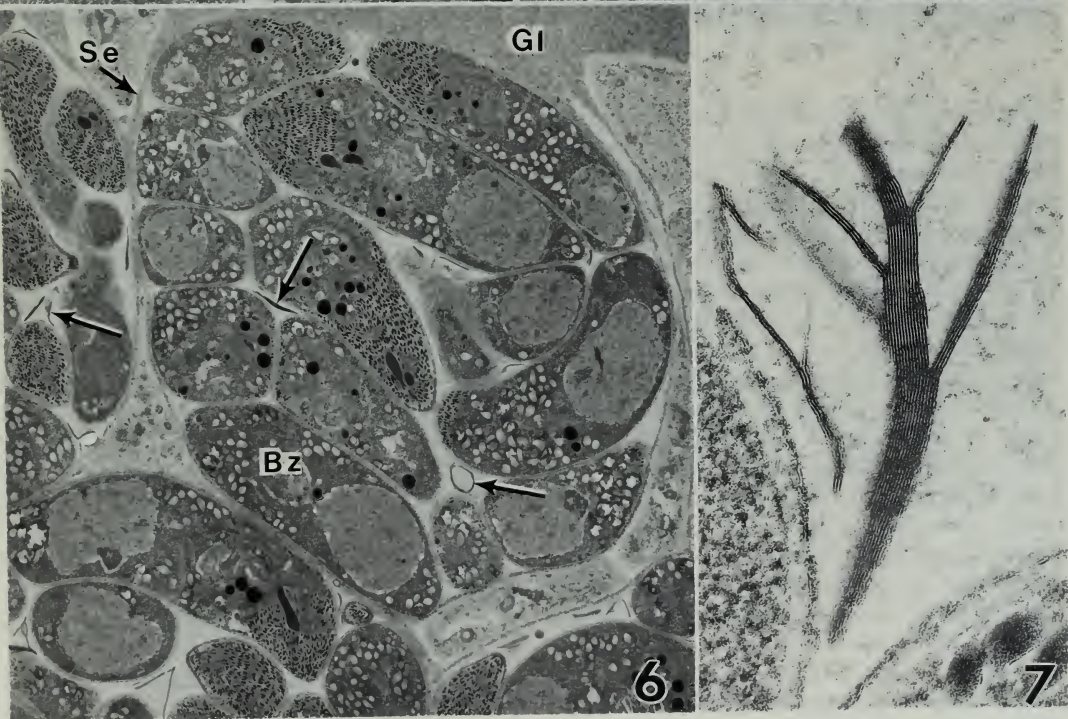
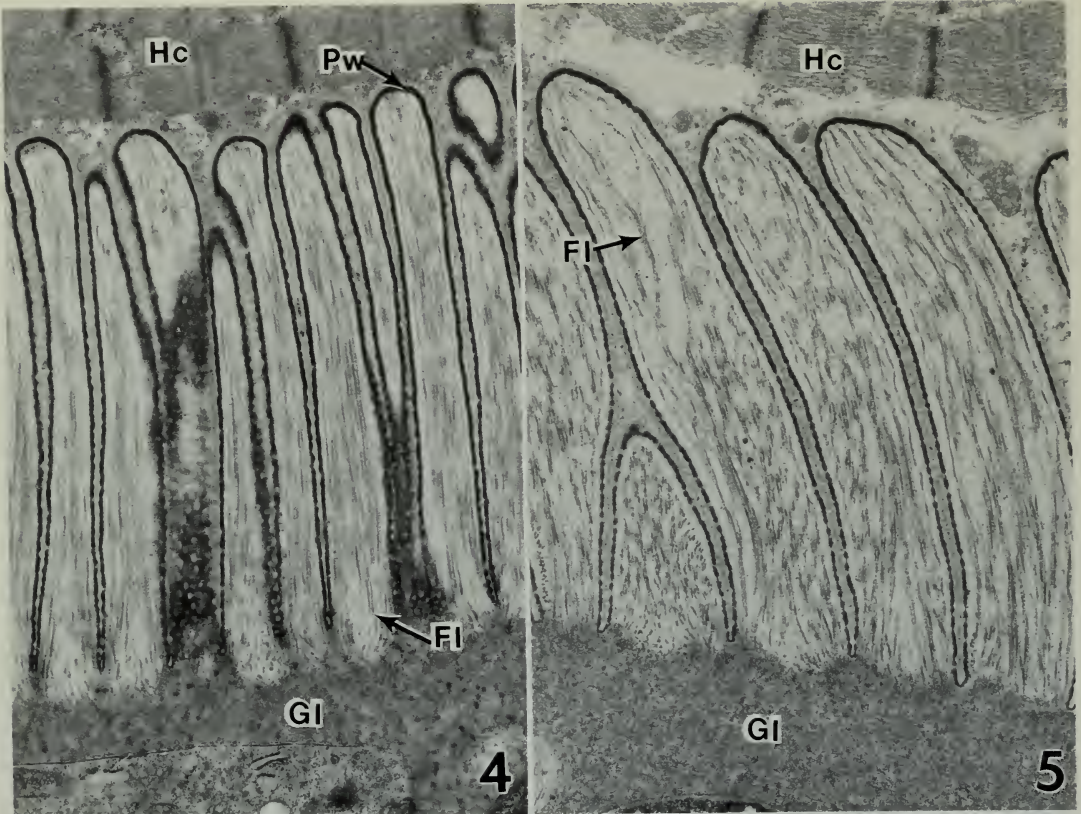
In both ages of sarcocysts, bradyzoites and metrocytes were arranged in compartments separated by septae (Fig. 6). Although metrocytes were more frequently located at the periphery, they were also located centrally. At 222 DPI, most compartments contained either bradyzoites or metrocytes but not both. The bradyzoites were loosely arranged within the compartments, whereas the metrocytes were usually tightly packed. Leaflets of membrane-like structures were also present in compartments containing bradyzoites in sarcocysts at 222 DPI, but not in those at 111 DPI (Fig. 7). At 222 DPI, metrocytes were present in peripherally located

compartments only. In both ages of sarcocysts, the granular layer between the villar projections and bradyzoites and metrocytes was similar in appearance and was 0.6–1.6  $\mu\text{m}$  thick.

#### DISCUSSION

The results of this study and those of Gestrich et al. (1975a) indicate that *S. hominis* is only mildly pathogenic for cattle. Calves fed  $10^6$  sporocysts did not have clinical signs except for fever, even though the sporocysts had been stored for 12 mo. Fever between 10 and 24 DPI was probably associated with the maturation of schizonts. The calves in the present study were necropsied at 10, 18, and 24 DPI to bracket the febrile period. Because of the limited number of sporocysts available, calves were fed a maximum of  $10^6$  sporocysts and only 5 calves were exposed. To find the schizogonic stages of *S. hominis*, tissues should be obtained at additional times and doses of more than  $10^6$  sporocysts may be necessary. In this respect, *S. hominis* appears to be similar to *S. hirsuta*. *Sarcocystis hirsuta* schiz-







onts were found in calves fed  $1.5$  and  $2.5 \times 10^6$  sporocysts but not  $10^6$  (Dubey, 1982).

Condemnation of beef containing macroscopic sarcocysts is a serious economic problem (Fayer and Dubey, 1986; Jensen et al., 1986; Böttner et al., 1987a, 1987b). Of the 3 species of *Sarcocystis* in cattle, the sarcocysts of *S. cruzi* always remain microscopic and are structurally distinctive (Mehlhorn et al., 1975a; Fayer and Dubey, 1986). Some of the macroscopic sarcocysts found in naturally infected cattle are those of *S. hirsuta* (Böttner et al., 1987a, 1987b). Whether *S. hominis* produces macroscopic sarcocysts has not been determined.

The differentiation of *S. hirsuta* sarcocysts from those of *S. hominis* is difficult at present because age-dependent ultrastructural differences in their sarcocyst walls have not been examined in detail (Gestrich et al., 1975b; Mehlhorn et al., 1975b). In the present study, although the *S. hominis* sarcocysts were mature at 111 DPI, the villar protrusions had changed at 222 DPI. The presence of leaflet-like structures in the sarcocyst at 222 DPI but not at 111 DPI has not been observed before in any sarcocyst.

#### ACKNOWLEDGMENTS

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FIGURES 4–7. Transmission electron micrographs of *Sarcocystis hominis* 111 days (Fig. 4) and 222 days (Figs. 5–7) after inoculation of sporocysts. 4. Portion of cyst wall showing relatively narrow villar projections containing uniformly distributed filaments (Fl); granular layer (Gl); host cell cytoplasm (Hc); primary cyst wall (Pw).  $\times 12,000$ . 5. Villar projections containing filaments (Fl) arranged in bundles; note that projections are slightly longer and about twice as wide as those in Figure 3; granular layer (Gl); host cell cytoplasm (Hc).  $\times 12,000$ . 6. Compartments within a sarcocyst containing bradyzoites (Bz) and membranous leaflets (arrows); granular layer (Gl); septa (Se).  $\times 3,900$ . 7. High magnification of membranous leaflets.  $\times 72,000$ .



# RESEARCH NOTES

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## Host Immunity Against Newborn *Trichinella spiralis* Larvae of Different Ages

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**ABSTRACT:** The infectivity of newborn *Trichinella spiralis* larvae of different ages was studied in normal rats. Newborn larvae collected after incubation of adult worms *in vitro* for 2, 12, or 24 hr were injected intravenously (i.v.) into normal AO rats in 3 separate recipient groups. All recipient rats developed strikingly similar numbers of muscle larvae 20 days later. The susceptibility to immunity by newborn larvae of different ages was also studied. No difference was found when degree of protection was compared by assessing muscle larvae burden or peritoneal anti-newborn larvae effects after injection of newborn larvae of different ages either i.v. or intraperitoneally into immunized recipient rats. We conclude that newborn larvae of any age up to 24 hr have similar infectivity in normal rats and are equally susceptible to anti-newborn larvae immunity *in vivo*.

The available evidence indicates that the *Trichinella spiralis* newborn larvae cuticle is the primary target of host immunity and that its molecular constituents are particularly dynamic during the first few hours after birth (Philipp et al., 1980, 1981; Parkhouse et al., 1981; Ortega-Pierres et al., 1984). It has been suggested that these cuticular changes influence susceptibility of the newborn larvae to immune effectors as assessed *in vitro*. The studies of Gansmuller et al. (1984) demonstrated that 2-hr larvae attracted eosinophils, whereas macrophages were the predominant cell attached to 20-hr larvae after incubation with peritoneal cells and immune serum *in vitro*. Binaghi et al. (1984) found 3-5-fold more muscle larvae in mice given a single injection of 20-hr-old newborn larvae than mice given 2-hr larvae. After incubation of larvae with immune serum and peritoneal cells *in vitro*, more young larvae were killed than older larvae. These results suggest that older larvae may be either more invasive or more resistant to immunity than young larvae. The biological relevance of these *in vitro* results to the *in vivo* expression of immunity to newborn larvae is not known.

Our previous studies on newborn larval migration and recirculation demonstrated that in rats more than 75% of newborn larvae have per-

manently left the circulation 3 hr after having entered it, irrespective of the age of the injected newborn larvae (Wang and Bell, 1986a). Because the time taken from birth to penetration of draining venules in the gut is less than an hour (Wang and Bell, 1986b), most newborn larvae are only exposed to the immune system for a maximum of 4 hr after their birth. Therefore, the biological significance of enhanced muscle larvae development by 20-hr-old newborn larvae is debatable. However, because a small proportion of newborn larvae do recirculate for 48 hr *in vivo*, it is evident that newborn larvae of all ages are exposed to the host immune system. In this report, host immunity against newborn larvae of different ages is examined in rats. The methods of obtaining muscle larvae, newborn larvae, assessment of immunity through comparison of muscle larvae burden, and detection of immunity in the peritoneal cavity have been described previously (Wang and Bell, 1986a, 1986b, 1987, 1988).

The first experiment determined the infectivity of newborn larvae of different ages in normal rats. Newborn larvae collected after incubation of adult worms *in vitro* for either 2, 12, or 24 hr were injected intravenously (i.v.) (15,000/rat) into 3 recipient groups consisting of 5 normal AO rats (6-8 wk old) per group. An average of  $9,476 \pm 930$  (2-hr larvae),  $8,706 \pm 512$  (12-hr larvae), and  $8,806 \pm 2,627$  (24-hr larvae) muscle larvae were detected 20 days after injection ( $P > 0.05$ , Student's *t*-test). The second experiment examined whether "young" or "old" newborn larvae were affected differently by host anti-newborn larvae immunity *in vivo*. Twenty-three rats were randomly assigned to 5 groups. On day 1, rats in groups 1, 2, and 4 were infected with 100 muscle larvae per os. On day 16, rats in groups 2 and 3 were injected i.v. with 26,700 newborn larvae collected 3 hr after incubation of adult worms *in vitro*. On the same day, rats in groups 4 and 5 were each challenged with the same num-

TABLE 1. Muscle larvae burden in rats after challenge with newborn larvae of different ages.

Groups	Treatment		Numbers of muscle larvae established (day 37)	Protection against challenge infection (%)
	ML (per os) immunization (day 1)	NBL (i.v.) challenge (day 16)		
1	100	—	23,433 $\pm$ 8,019	
2	100	26,700 (3 hr old)	33,600 $\pm$ 7,171	48.5
3	—	26,700 (3 hr old)	19,375 $\pm$ 1,548	
4	100	26,700 (24 hr old)	31,400 $\pm$ 8,104	58.3
5	—	26,700 (24 hr old)	19,125 $\pm$ 3,449	

Data represent means  $\pm$  1 SD for 4–5 rats per group.

ber of newborn larvae that were collected after *in vitro* maintenance of adult worms for 24 hr. Group 1 rats were not challenged and were immunization controls, whereas group 3 and 5 rats were controls for the challenge infection. As shown in Table 1, no difference ( $P > 0.50$ ) was found in the number of muscle larvae that developed in the 2 immunized groups nor in 2 control groups, irrespective of whether challenge newborn larvae were 3 or 24 hr old. As might be expected from these results, the degree of protection against the challenge infection was essentially the same for both groups, i.e., 48.5% for 3-hr larvae and 58.3% for 24-hr larvae.

Most of the *in vitro* data on susceptibility of newborn larvae of different ages to immunity were generated using peritoneal cells. To determine whether immunity, as detected directly in the peritoneal cavity, might be different from systemic immunity, the following experiment was conducted. Rats (31) were randomized into 8 paired groups, 4 immunized and 4 control. On day 1, 2,000 muscle larvae were given to rats in the 4 immunized groups. After 15 days, immunized rats were injected intraperitoneally (i.p.) with either 1,020, 7,380, 15,000, or 20,000 newborn larvae (groups 1–4) that were collected at 1, 3, 10, or 24 hr after incubation of adult worms. The 4 control groups were infected identically with their test groups. The peritoneal fluid of these rats was examined 6 hr after larval injection. As shown in Figure 1, the average larval recovery in immunized rats ranged from 10 to 22%, which was significantly lower than the controls (43–56%), and this was independent of newborn larval age when injected. The percentage of

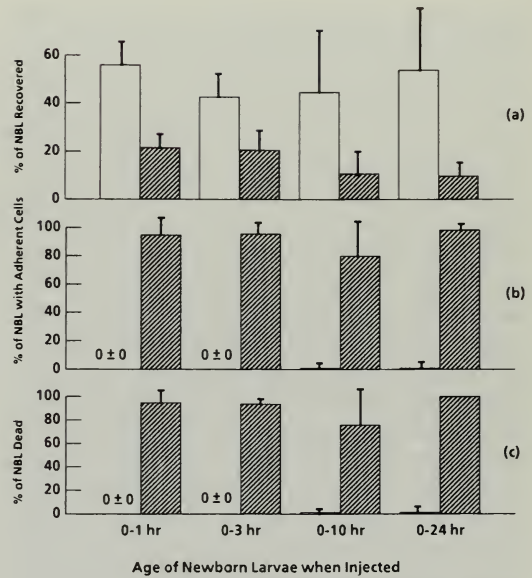


FIGURE 1. Anti-newborn larvae immunity in rats after i.p. challenge with newborn larvae of different ages. Rats in 4 immune groups (▨) were infected with 2,000 muscle larvae 15 days previously. Rats in individual control (□) and immune groups were injected i.p. with 1,020, 7,380, 15,000, or 20,000 newborn larvae collected after *in vitro* incubation of adult worms for 1, 3, 10, or 24 hr. The peritoneal fluid of these rats was examined 6 hr after newborn larvae injection. Data represent means  $\pm$  1 SD for 3–4 rats per group. All immune groups are significantly different from the controls but they do not differ ( $P > 0.05$ ) from each other.

recovered larvae that had adherent cells, or were dead, was 0–1%, in all control rats. However, both parameters increased dramatically in all immunized rats (~80–100%) and were not influenced by the age or dose of newborn larvae given. Even though the results obtained from rats in individual immunized groups were all significantly different ( $P < 0.05$ ) from their individual controls, they were not different ( $P > 0.05$ ) from each other. Similar results were obtained for number of larvae killed or with adherent cells when these were measured 2 hr after injection of newborn larvae i.p. (data not shown).

These experiments indicate that newborn larvae of different ages have the same infectivity in normal rats. This is demonstrated by the fact that all normal recipient rats developed strikingly similar numbers of muscle larvae despite the differences in age of injected newborn larvae (experiments 1 and 2). We do not know why these results differ from those of Binaghi et al. (1984). The mice used in their experiments may be more



permissive to the invasion of muscle cells by older newborn larvae and less so towards younger larvae. However, the method used by Binaghi et al. to incubate adult worms *in vitro* also differed from that used here. For example, in their experiments, the same batch of adult worms was incubated repeatedly *in vitro* to obtain 2-hr newborn larvae, whereas 20-hr newborn larvae were collected after overnight culture. Under these conditions, damage or premature aging of the adult worms may contribute to variation in larval infectivity in normal mice. We have not attempted to examine this question.

Secondly, our data demonstrate that newborn larvae of different ages are equally vulnerable to anti-newborn larvae immunity *in vivo*. As mentioned above, we have demonstrated that a small proportion of newborn larvae remain outside muscle cells 24 hr or more after they enter the circulation (Wang and Bell, 1986a). Because of this, it seems likely that all cuticular antigen variants of newborn larvae up to 24 hr old are naturally exposed to the host immune system and are likely to be recognized immunologically. The fact that immunity was evident against challenge with either 3-hr or 24-hr newborn larvae (Table I) suggests that immunity is equally effective in rats against young and old larvae. Similar conclusions were reached when peritoneal cavity immunity was measured directly after injection of newborn larvae i.p. into immunized and control rats (Fig. 1). In immunized rats, larval recovery went down, and cell adherence and larval mortality went up irrespective of the age and dose of newborn larvae given. The fact that the results obtained from individual immunized groups were not significantly different ( $P > 0.05$ ) from each other further strengthens the second point and indicates that immune recognition involves antigens expressed by newborn larvae of all biologically relevant ages. It is important to point out that the immunity detected in the peritoneal cavity is a specific immunological response. Intraperitoneal injection with thioglycollate failed to elicit any significant cell adherence on the larval cuticle, and no larval killing by the cellular exudate was observed (Wang and Bell, 1988).

In summary, the experiments presented here demonstrate that newborn larvae of any age up to 24 hr have similar infectivity in normal rats and are equally vulnerable to attack by host immune effectors *in vivo*.

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## Free-living Amoebas and Cold-blooded Animals

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**ABSTRACT:** A total of 110 reptiles and 40 amphibians were examined for free-living amoebas. Eighty-two clones of amoebas were isolated by culture of gut contents—30 of them belonged to the genus *Acanthamoeba* and 3 to the genus *Naegleria*.

Since 1940 the presence of free-living amoebas in the intestine of cold-blooded animals has been reported by several authors (Lobeck, 1940; Franke and Mackiewicz, 1982). In our case, some of the amoebas found in different reptiles and amphibians showed morphological differences from the well-known parasitic species. Therefore, vegetative forms of free-living amoebas were observed and isolation and identification were then attempted.

Protozoa were obtained by culturing the gut contents of 150 specimens of reptiles and amphibians belonging to *Gallotia galloti galloti* Duméril and Bibron, 1839 (lizard, 10 specimens), *Tarentola delalandii* Steindachner, 1891 (gecko, 50 specimens), *Chalcides viridanus viridanus* Steindachner, 1891 (seps, 50 specimens), and *Rana perezi* Hotz, 1974 (frog, 40 specimens). A nonnutrient agar supplied with a young culture of *Enterobacter cloacae* was used as culture medium. The plates were incubated at 25 C for 14 days. We observed the presence of live amoebas during this period. At the end of the 14 days, negative cultures were rejected and the positive ones were isolated as clones (Molet and Kremer, 1976). Amoebas were maintained by passages in the same medium. Identification was carried out according to Page (1976).

The results of isolation are shown in Table I. The following strains were identified: In *Gallotia galloti galloti*: 1 *Acanthamoeba triangularis*, 5 *Acanthamoeba* spp. (*polyphaga-quina-lugdunensis* complex), 1 *Echinamoeba exundans*, 1 *Platyamoeba placida*. In *Tarentola delalandii*: 1 *Acanthamoeba lenticulata*, 1 *Acanthamoeba triangularis*, 3 *Acanthamoeba* spp. (*polyphaga-quina-lugdunensis* complex), 2 *Acanthamoeba* spp., 1 *Adelphamoeba galeacystis*, 3 *Filamoeba*

*nolandi*, 6 *Hartmannella* spp., 2 *Naegleria gruberi*. In *Chalcides viridanus viridanus*: 3 *Acanthamoeba* spp. (*polyphaga-quina-lugdunensis* complex), 11 *Acanthamoeba* spp., 2 *Adelphamoeba galeacystis*, 10 *Filamoeba nolandi*, 4 *Hartmannella vermiformis*, 2 *Hartmannella* spp., 1 *Pessonnella marginata*, 4 *Platyamoeba stenopodia*, 3 *Vahlkampfia enterica*, 3 *Vahlkampfia* spp. In *Rana perezi*: 3 *Acanthamoeba* spp. (*polyphaga-quina-lugdunensis* complex), 3 *Hartmannella vermiformis*, 1 *Naegleria* sp., 1 *Vahlkampfia avara*, 4 *Vahlkampfia enterica*.

It is necessary to consider whether the presence of amoebas in reptiles and amphibians is by chance or whether it is a true colonization. Observation of gut contents in this study revealed many vegetative forms of common intestinal ciliates (i.e., *Balantidium* and *Nyctoterus*), cysts of free-living ciliates (i.e., *Colpoda*), and vegetative forms and cysts of the same amoebas that were isolated.

Fernandez-Galiano et al. (1986) believe that the presence of free-living ciliates is a chance finding supported by their very resistant cyst wall. However, vegetative forms of amoebas are actually found in the course of the digestive tract, thus we believe that this is a true colonization. Nevertheless, pathogenicity of amoebas for these cold-blooded animals did not seem to be important because they are not as pathogenic as *Entamoeba invadens*.

With regard to the number of amoebas that have been isolated in this work, our results are similar to those of other investigators, except that our results yielded higher percentages. For example, Ciurea-Van Saanen (1980) found that only 21.4% of reptiles were positive. Also, in comparison to other studies, the culture medium used in the present study is more effective because it reduces growth of contaminants. Finally, the amoebas found in this study and those found by other investigators are very common. *Hartmannella*, *Acanthamoeba*, *Naegleria*, and *Vahlkampfia* (Frank and Bosch, 1972; Bosch, 1973).



**TABLE 1.** *Prevalence of amoebic strains isolated from various hosts.*

Species	No. specimens	% Positive specimens	No. isolated strains
<i>Gallotia galloti galloti</i>	10	80	8
<i>Tarentola delalandii</i>	50	36	19
<i>Chalcides viridanus viridanus</i>	50	64	43
<i>Rana perezi</i>	40	20	12

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Digenetic Trematodes: Multiplication of the Intramolluscan Stages in Some Species Is Potentially Unlimited

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**ABSTRACT:** By carrying out chain-transplantations of intramolluscan stages of digeneans from infected to uninfected snails it has been demonstrated that a great number of generations of these stages can follow one another. In Echinostomatids the succession of redial generations potentially seems to be unlimited.

In digenetic trematodes that use molluscs as intermediate hosts the actual number of generations of the intramolluscan stages (either rediae or sporocysts) has never been determined exactly. The different generations are indistinguishably mixed. Now, by means of a transplantation procedure in which a single (newborn) redia or sporocyst is transferred from an infected to an uninfected snail, the generations can be analyzed with regard to their rank. For at least 2 echinostomatid species it appears that the succession of redial generations is potentially unlimited.

*Isthmiophora melis* (Echinostomatidae)

The first species in which this was observed is the intestinal fluke *Isthmiophora melis*, a parasite

of mustelids and man. Our redial strain has passed 45 transplantation steps in 7 yr in the laboratory without loss of the ability to produce generative adults and infective miracidia (Dönges and Götzelmann, 1975). The transplantation is performed by inserting a microprecision syringe fitted with a glass needle of 50  $\mu$ m inner diameter through the foot tissue, deeply into the lymphatic spaces of the visceral sac. The technique is simple and is described by Dönges (1968). How proof of the minimum number of generations in a transplantation chain is obtained is also explained by Dönges (1971). Only newborn rediae (smallest size class) were transferred.

*Echinoparyphium aconiatum* (Echinostomatidae)

We serially transplanted a redial strain of *Echinoparyphium aconiatum*, an intestinal fluke of water fowl, in its first intermediate host, *Lymnaea stagnalis*, for 5,844 days (=16 yr, 4 days). The transplantation strain was initiated with rediae from a naturally infected specimen of *L.*

*stagnalis* taken from Lake Neusiedl, Austria. In 1 uninterrupted transplantation chain, 77 sub-transplantations were performed at a mean interval of 76 days. After the transfer of newborn rediae of *E. aconiatum* to an uninfected, laboratory-reared juvenile specimen of *L. stagnalis* (shell length 18–22 mm), the first newborn rediae of the next generation appear after 13–18 days (at 23°C). From a generation frequency of 1 in 15.5 days it can be calculated that the total number of generations through which the redial strain passed uninterruptedly and without any inserted bisexual reproduction (in a final host) is 377.

#### *Fasciola gigantica* (Fasciolidae)

An analogous experiment was performed with *Fasciola gigantica* and its intermediate host *Lymnaea natalensis*. The redial transplantation strain was started with a miracidial exposed snail and yielded, after 6 transplantation steps within a period of 276 days, about 20 redial generations. No loss of viability and no loss of the ability to produce normal cercariae and metacercariae were observed after this period.

#### *Schistosoma mansoni* (Schistosomatidae)

In a study on the intramolluscan development of *Schistosoma mansoni*, Lang (1982) transplanted daughter sporocysts. In one experiment he established 6 generations of daughter sporocysts succeeding one another. In *Schistosoma* the number of generations of these intramolluscan stages may be potentially unlimited. Generally, in digeneans that adhere to the sporocyst devel-

opment pattern (e.g., *Schistosoma*) the tendency to produce successive numbers (more than 2) of intramolluscan generations is less than in digeneans following the redial development pattern (e.g., echinostomatids).

#### Conclusions

These experiments demonstrate that in some species of digeneans intramolluscan development can proceed *ad infinitum*. The transplantation chain simulates a snail of unlimited size and life span. Thus, there appears to be no genetically programmed end to redial multiplication.

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## Lizard Hemoparasites from "Los Tuxtlas" Tropical Biology Station, Veracruz, Mexico

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**ABSTRACT:** A total of 386 lizards from a tropical rain forest in Veracruz, Mexico, representing the families Gekkonidae, Iguanidae, Xantusiidae, Scincidae, and Teiidae, were examined for hemoparasites on 3 oc-

casions between March 1985 and May 1986. Hemoparasites were observed in 3 iguanid species: *Sceloporus variabilis* was infected with *Plasmodium floridense* and *Trypanosoma servi*, *Corytophanes her-*



*nandezi* with *T. serveti* and microfilariae of *Piratuba* sp., and *Anolis tropidonotus* with all of these parasites as well as hemogregarine gamonts. Adult *A. tropidonotus* had a higher prevalence of infection with *P. floridense* but not with *T. serveti* when compared with juveniles. Prevalence of *P. floridense* infection in adult *A. tropidonotus* bled during the dry season was higher in males than in females. Adult male and female filariae of the genus *Piratuba* were recovered from the heart chambers upon necropsy of an adult *C. hernandezi*, and additional worms were recovered from the same location from museum specimens. In contrast, adult filariae of the genus *Piratuba* have previously been recovered from the lungs of Australian varanid lizards and the general body cavity of neotropical iguanid and teiid lizards.

Lizards were sampled for hemoparasites on 3 occasions between March 1985 and May 1986 at the "Los Tuxtlas" Tropical Biology Station, Veracruz, Mexico. "Los Tuxtlas" is located within the northernmost zone of tropical rain forest habitat in North America, 33 km northeast of Catemaco and 4.5 km from the Gulf of Mexico. Lizards were collected in the late rainy season (March–April 1985), mid-rainy season (August–September 1985), and finally the brief dry season (May 1986) experienced at "Los Tuxtlas."

A total of 386 lizards were captured by hand or using drift fences (Vogt and Hine, 1977) and identified with the help of resident herpetologists at the biology station. The following species were sampled: GEKKONIDAE—*Sphaerodactylus glaucus glaucus* Cope (2); IGUANIDAE—*Anolis barkeri* Schmidt (20), *A. tropidonotus tropidonotus* Bocourt (281), *Basiliscus vittatus* Wiegmann (6), *Corytophanes hernandezi* (Wiegmann) (11), *Iguana iguana* (Linnaeus) (1), *Sceloporus internasalis* Smith and Bumzahem (1), and *S. variabilis variabilis* Wiegmann (37); XANTUSIIDAE—*Lepidophyma pajapanensis* Werler (1); SCINCIDAE—*Eumeces sumichrasti* (Cope) (2), *Sphenomorphus cherriei stuarti* (Smith) (1); and TEIIDAE—*Ameiva undulata amphigramma* Smith and Laufe (19). Snout–vent lengths (SVL's) were measured using calipers, and SVL's of *A. tropidonotus* were plotted on a histogram; sex was also determined. Most lizards were bled from toe clips for thin blood films and subsequently released; *A. tropidonotus* having an SVL <31 mm were sacrificed by cervical dislocation, and blood was obtained from the heart, because insufficient blood is obtained from toe clips of lizards of this size.

Blood films were dried, fixed in absolute methanol for 10 min, and stained with 3% (v/v) Giem-

sa stain in phosphate-buffered water, pH 7.0, for 1 hr. Slides were scanned for extracellular parasites at 100× magnification, and for intracellular parasites for a minimum of 10 min at 400× magnification. Slides without sufficient blood to allow observation for this time period were not included in this study. When malaria parasites were observed, it was noted whether schizonts, gametocytes, or both were present. Microfilariae were measured at 1,000× magnification.

An adult *C. hernandezi*, positive for microfilariae, was sacrificed and necropsied; heart, lungs, carcass, and skin were dissected in physiological saline and examined immediately and after soaking overnight. Worms were killed and fixed in glacial acetic acid, stored in 70% ethanol plus 5% glycerine, and cleared in glycerine. The hearts of 7 alcohol-preserved museum specimens of *C. hernandezi* from "Los Tuxtlas" were subsequently cut open and worms processed as above.

*Sceloporus variabilis* was infected with *Plasmodium floridense* Thompson and Huff and *Trypanosoma serveti* Peláez and Streber; *C. hernandezi* with *T. serveti* and microfilariae of *Piratuba* sp. Lent and Freitas; *A. tropidonotus* with all these parasites as well as hemogregarine gamonts. No parasites were seen in blood films of the remaining lizard species examined (Table I). Schizonts of *P. floridense* were observed in 60% (n = 20) of infected *A. tropidonotus* examined during the mid-rainy season and in 52% (n = 79) during the dry season.

Using histograms, juvenile *A. tropidonotus* were considered to be those lizards with an SVL <31 mm for those collected in August–September 1985. This cutoff point was also used for lizards obtained in May 1986, although the 2 populations were less clearly appreciated in histograms, perhaps due to growth or mortality of juvenile lizards born during the previous summer. Adult *A. tropidonotus* had a higher prevalence of *P. floridense* than juveniles, but this pattern was not seen consistently with *T. serveti* infection (Table II).

A mature female and the posterior end of a male filaria were found in the heart chambers of the necropsied lizard; no nematodes were recovered from the body cavity, lungs, carcass, or skin. Five museum specimens also contained male and/or female filariae in the heart chambers. Morphology of the adults and the microfilariae was consistent with that of the genus *Piratuba*. Microfilariae had cephalic hooks, were sheathed, and from 31 to 57% of the posterior

TABLE I. Prevalence of hemoparasites in lizards from "Los Tuxtlas," Veracruz, Mexico, 1985-1986.

Lizard sp.	n	% Infected with:*			
		P.f.	T.s.	Hg.	Mf.

March-April 1985					
<i>Anolis tropidonotus</i>	14	21	14	0	0
<i>Corytophanes hernandezi</i>	5	0	0	0	40
<i>Sceloporus internasalis</i>	1	0	0	0	0
<i>Sceloporus variabilis</i>	2	0	0	0	0
<i>Ameiva undulata</i>	5	0	0	0	0
August-September 1985					
<i>Sphaerodactylus glaucus</i>	1	0	0	0	0
<i>Anolis tropidonotus</i>	72	28	10	0	0
<i>Basiliscus vittatus</i>	6	0	0	0	0
<i>Corytophanes hernandezi</i>	1	0	0	0	0
<i>Iguana iguana</i>	1	0	0	0	0
<i>Sceloporus variabilis</i>	31	10	19	0	0
<i>Eumeces sumichrasti</i>	1	0	0	0	0
<i>Ameiva undulata</i>	2	0	0	0	0
May 1986					
<i>Sphaerodactylus glaucus</i>	1	0	0	0	0
<i>Anolis barkeri</i>	20	0	0	0	0
<i>Anolis tropidonotus</i>	195	41	10	0.5	1.5
<i>Corytophanes hernandezi</i>	5	0	20	0	20
<i>Sceloporus variabilis</i>	4	50	25	0	0
<i>Lepidophyma pajapanensis</i>	1	0	0	0	0
<i>Eumeces sumichrasti</i>	1	0	0	0	0
<i>Sphenomorphus cherriei</i>	5	0	0	0	0
<i>Ameiva undulata</i>	12	0	0	0	0

\* P.f. = *Plasmodium floridense*; T.s. = *Trypanosoma serveti*; Hg. = hemogregarines; Mf. = microfilariae of *Piratuba* sp.

end was folded back upon the body within the sheath. They ranged in total body length from 69 to 81  $\mu$ m and in maximum body width from 5 to 8  $\mu$ m ( $n = 30$ ). Microfilariae seen in 3 of 281 *A. tropidonotus* were identical with those harbored by *C. hernandezi*.

The finding of *P. floridense* in Veracruz iguanids is not surprising. Infections in various *Sceloporus* and *Anolis* spp. have been reported previously from the southeastern United States (Thompson and Huff, 1944; Goodwin, 1951; Jordan, 1964; Jordan and Friend, 1971; Telford, 1978), Central America (Telford, 1974, 1977; Guerrero et al., 1977), and several Caribbean islands (Ayala, 1975; Telford, 1975; Guerrero and Pickering, 1984). Our finding that adult *Anolis* had a higher prevalence of *P. floridense* infection than juveniles (Table II) correlates well with other studies (Goodwin, 1951; Guerrero et al., 1977; Guerrero and Pickering, 1984; Rand et al., 1984). Male anoles have been reported to have a higher prevalence of *P. floridense* than females in Panama (Guerrero et al., 1977; Rand et al., 1984), as was observed in the dry season in "Los Tuxtlas" (Table II).

TABLE II. Size- and sex-related prevalence of malarial and trypanosome infections in *Anolis tropidonotus* from "Los Tuxtlas," Veracruz, Mexico, in wet (August-September 1985) and dry (May 1986) seasons.

Season collected	Adult ♂	Adult ♀	Juvenile
n* (% infected with <i>Plasmodium floridense</i> )			
Wet	17 (35)	23 (48)	32 (9)
Dry	106 (49)	72 (38)	17 (0)
n (% infected with <i>Trypanosoma serveti</i> )			
Wet	17 (24)	23 (13)	32 (0)
Dry	106 (11)	72 (7)	17 (12)

\* n = number of lizards examined.

*Trypanosoma serveti* has been described from *Sceloporus* and *Anolis* spp. in Veracruz, Mexico (Peláez and Streber, 1955), Panama (Guerrero et al., 1977), and Peru (Guerrero and Ayala, 1977). There is less known concerning the ecology of this parasite. We observed slightly more infections in adult male *A. tropidonotus* than in females (Table II).

Our finding of *Piratuba* sp. is of interest not only because this is a new host record for *Corytophanes*, but also because in all previous reports from the western hemisphere, adults were found in the general body cavity of neotropical iguanid and teiid lizards rather than in the heart (Lent and Freitas, 1941; Peláez and Pérez-Reyes, 1958, 1960; Sonin and Baruš, 1968; Bain, 1974; Vicente, 1981). Mackerras (1962) recovered *Piratuba* spp. adults from the lungs of Australian varanid lizards. Although Peláez and Pérez-Reyes (1960) describe *Piratuba lanceolata* from *Sceloporus teapensis* Günther from San Andres Tuxtla, Veracruz, microfilariae from nearby "Los Tuxtlas" were nearly twice as long and displayed considerably greater doubling over within their sheaths. They more closely resembled those of *Piratuba prolifica* from *Sceloporus mucronatus* Günther from Guerrero, Mexico (Peláez and Pérez-Reyes, 1958). In the only previous report of filarial infection of *Corytophanes* sp., Telford (1977) found unidentified microfilariae in blood films of *C. cristatus* in Panama.

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Rodolfo Dirzo for helping arrange the collecting trips; Mr. Barry G. Campbell and Drs. Robert G. Yaeger and Richard C. Vogt for their expert scientific advice; and Dr. Sam R. Telford, Jr., for confirming our identification of *Plasmodium floridense* in *Anolis tropidonotus*. This study was funded by Tinker Foundation Inc., Mesoamerican Ecology Institute, Tulane University, New Orleans, Louisiana 70118.

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## ***Sarcocystis sigmodontis* n. sp. from the Cotton Rat (*Sigmodon hispidus*)**

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**ABSTRACT:** *Sarcocystis* sarcocysts were found in 3 of 4 cotton rats (*Sigmodon hispidus*) from Atlanta, Georgia. Sarcocysts were several centimetres long and were present only in skeletal muscles. The sarcocyst wall appeared thin ( $<1\ \mu\text{m}$ ), with minute projections in the light microscope. By transmission electron microscopy, the sarcocyst wall had  $0.6\text{--}1.0 \times 0.21\text{--}0.36\text{-}\mu\text{m}$  villar protrusions without microtubules. The metrocytes were  $6.5 \times 3.8\ \mu\text{m}$ , and the bradyzoites were  $8 \times 2.7\ \mu\text{m}$ . The sarcocysts were not infectious for dogs and cats. The parasite was named *Sarcocystis sigmodontis* because it differed from all sarcocysts in rodents.

Virtually all herbivores including mammals, marsupials, birds, and poikilothermic animals have been found infected with asexual stages of *Sarcocystis*. We report *Sarcocystis* infection in cotton rats because there is no report of *Sarcocystis* infection in this host.

During the course of epidemiological investigation of an outbreak of toxoplasmosis in humans associated with a riding stable near Atlanta, Georgia (Teutsch et al., 1979), cotton rats (*Sigmodon hispidus*) trapped in and around the stable were examined for *Toxoplasma* and *Sarcocystis* infection. Three of 4 cotton rats were heavily infected with *Sarcocystis*. The affected animals appeared clinically normal.

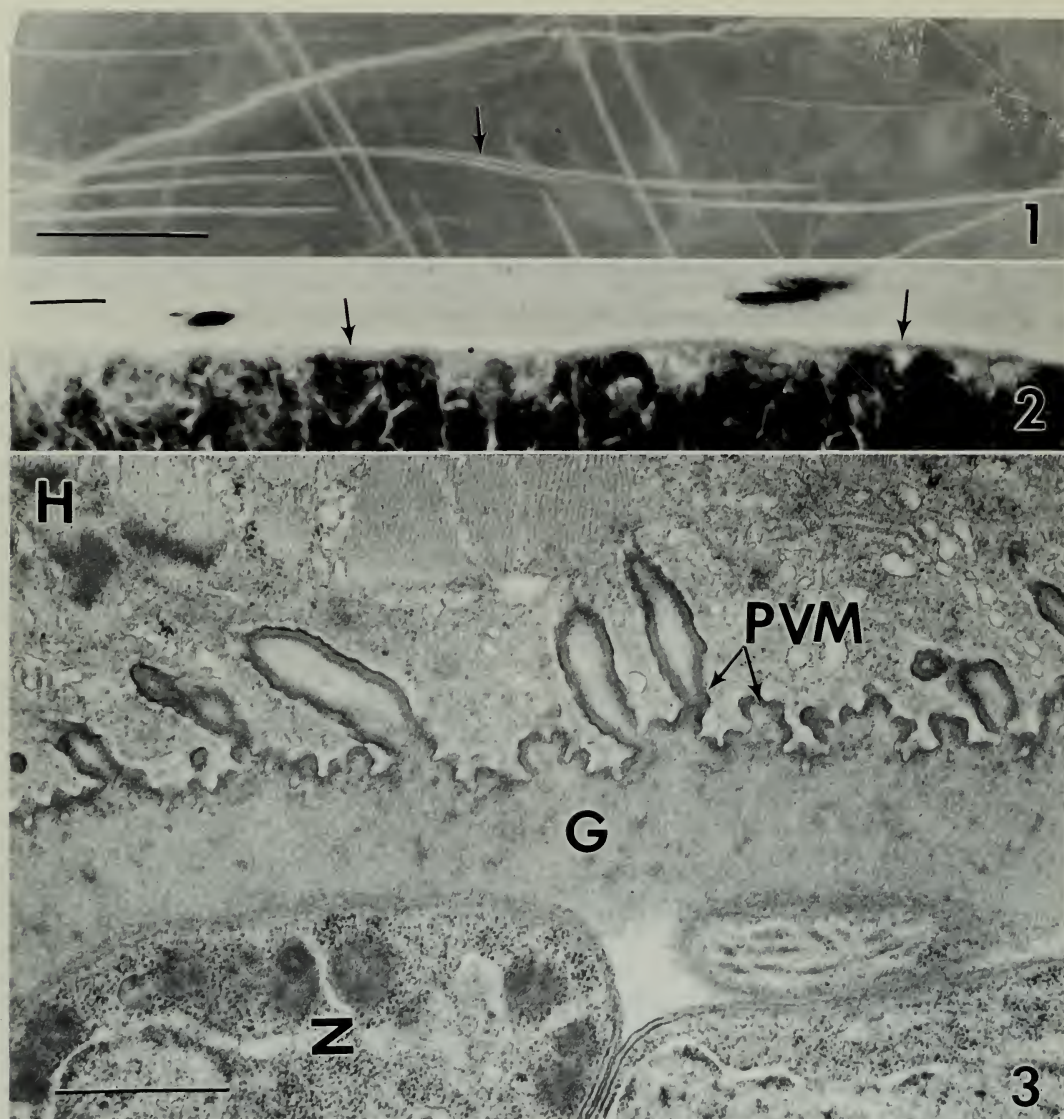
Carcasses from the 2 cotton rats (1 and 3) containing macroscopic sarcocysts were homogenized in a blender and fed to specific pathogen-free cats and dogs. Tissues from rat 1 were fed to 1 dog and 3 cats and tissues from rat 3 were fed to another dog and another cat. The cats and dogs had been raised in the laboratory from birth and had never eaten uncooked meat until used in the experiment. The feces of the dogs and cats were examined daily using sugar flotation for *Sarcocystis* sporocysts or oocysts for a period of 3-4 wk after the animals had been fed rat tissues. The dogs and cats were euthanatized after termination of the fecal examination period, and their intestines were examined for trapped sporocysts of *Sarcocystis*. For *Sarcocystis* exami-

nation, mucosa and submucosa were scraped from the small intestine, homogenized in a blender, and incubated in 5.25% sodium hypochlorite (undiluted bleach) solution for 30-60 min in an ice bath. The digested intestinal material was centrifuged at 400 g for 10 min, and the sediment was examined for *Sarcocystis* sporocysts as described by Dubey (1980). By this method one can recover a majority of *Sarcocystis* from the intestinal tissue.

Portions of brain, heart, lungs, liver, spleen, diaphragm, and abdominal and other skeletal muscles were fixed in 10% neutral buffered formalin. Paraffin sections were cut at  $5\ \mu\text{m}$ , stained with hematoxylin and eosin, and examined. Portions of macroscopic sarcocysts from the abdomen were fixed in 2% buffered glutaraldehyde, processed for transmission electron microscopy, and examined in an electron microscope (Sheffield and Melton, 1966).

The sarcocysts were several centimetres long and up to  $140\ \mu\text{m}$  wide (Fig. 1). They were found only in sections of skeletal muscle. By light microscopy, the sarcocyst wall was thin ( $<1\ \mu\text{m}$ ) and smooth to spiny in sectioned skeletal muscle (Fig. 2). Ultrastructurally, the parasitophorous vacuolar membrane (PVM) was lined by an electron-dense layer (EDL) of uniform thickness; the PVM plus the electron-dense layer was about 40 nm thick (Fig. 3). The primary cyst wall (PCW) was thrown into small bleblike structures throughout the sarcocyst wall; the EDL was interrupted at irregular intervals in these blebs. At irregular intervals, the PCW was folded into straight to sloping villar protrusions. These villar protrusions were of uneven length ( $0.6\text{--}1.0\ \mu\text{m}$ ,  $n = 5$ ) and width ( $0.21\text{--}0.36\ \mu\text{m}$ ,  $n = 7$ ); there were no microtubules in the villar protrusions. The ground substance (G) of the sarcocyst wall consisted of an electron-lucent amorphous layer  $0.6\text{--}1.2\ \mu\text{m}$  thick ( $n = 10$ ), and continued into the interior of the sarcocyst as septa. Metrocytes





FIGURES 1–3. Sarcocysts in skeletal muscles of a cotton rat. 1. Macroscopic threadlike sarcocysts in abdominal muscles. Unstained. Bar = 5 mm. 2. Section of a sarcocyst. Arrows point to thin sarcocyst wall with minute striations. Bar = 10  $\mu$ m. 3. Transmission electron micrograph of a sarcocyst in a cotton rat. Parasitophorous vacuolar membrane (PVM) is extended into host cell (HC). Zoites (Z) are located at the base of the sarcocyst wall. Bar = 1  $\mu$ m.

and bradyzoites were arranged in packets separated by granular septa. Metrocytes were located both centrally and at the periphery of the sarcocyst; they contained organelles found in other species of *Sarcocystis*. The metrocytes were  $6.5 \times 3.8 \mu\text{m}$  ( $5.0\text{--}7.0 \times 2.8\text{--}4.9 \mu\text{m}$ ,  $n = 10$ ). Bradyzoites were  $2.7 \mu\text{m}$  ( $2.2\text{--}3.4$ ,  $n = 22$ ) wide. Longitudinally cut bradyzoites were  $7.9 \mu\text{m}$  ( $6.5\text{--}9.5 \mu\text{m}$ ,  $n = 11$ ) long.

*Sarcocystis* sporocysts or oocysts were not found in the feces or intestines of dogs and cats.

*Sarcocystis* species are generally host specific; at least 19 species of *Sarcocystis* are known to infect rodents. However, none has been reported from the cotton rat (Levine, 1986). The structure of the sarcocyst wall appears to be a reliable criterion for distinguishing *Sarcocystis* species within a given host. The structure of the sarcocyst

wall in the cotton rat is structurally different from any of the *Sarcocystis* species in other rodents (Dubey et al., 1988). Therefore, the sarcocysts in the cotton rat were named *Sarcocystis sigmodontis*.

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## Cage Design for the Confinement of Deer and Goats Infested with Ectoparasites

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**ABSTRACT:** The design for a cage for the confinement of white-tailed deer (*Odocoileus virginianus*) and goats (*Capra* sp.) for use in ectoparasite studies is presented. The cage was successfully used for the infestation of these hosts with *Boophilus annulatus* and may be useful for host/parasite studies using similar hosts and other ectoparasites. The cage will accommodate mature hosts with adequate space for normal grooming activity. A detailed description of materials and methods for the assembly of the cage is provided in the text.

Ectoparasite studies of wildlife and domestic animals in laboratory settings often require a suitable method for confinement of the host that minimally alters the life cycle of the parasite or the behavior of the host. To study the host/parasite relationships of *Boophilus annulatus* and white-tailed deer (*Odocoileus virginianus*) and goats (*Capra* sp.), we needed a cage to confine these hosts during the on-host parasitic period of the tick that would provide adequate space for normal host grooming activity. The cage should also facilitate other research needs such as obtaining blood samples, observation of tick development, and retrieval of detached female ticks. The health and comfort of the host, prevention of injury, sufficient air circulation, and easy cleaning were major prerequisites for the

cage design. We found commercial calf stalls and stanchions (Nasco Products, Inc., Fort Atkins, Wisconsin 55538) as well as a design for an elevated calf stall for rearing ticks (Sutherst et al., 1978) that could be modified to hold tick-infested goats under restrained conditions. However, they were not suitable for use with unrestrained white-tailed deer or goats. A cage that met the requirements for our studies with *B. annulatus* and alternate hosts was designed and constructed as described below.

The cage was metal and plywood with overall dimensions of 1.22 × 0.61 × 1.37 m. The following materials and quantities were used: 12.80 m of 3.81 × 3.81-cm angle iron; 8.53 m of 1.91-cm channel iron; 2.23 m<sup>2</sup> of 1.27-cm expanded metal for sides and top; 0.74 m<sup>2</sup> of 3.81 expanded metal for flooring; 0.61 m of 1.91-cm (inside diameter) steel pipe to support flooring; 2.44 × 1.22-m sheet of 1.91-cm marine grade plywood; 2 elastic cords ca. 30 cm in length, 4 eye hooks; 5 screen-door-type handles; 2 screen-door-type hooks; 19 3.18-cm round-headed stove bolts with washers and nuts; 1 rubber mat ca. 1.07 × 0.46 m; rust-proofing paint; and 1 pet waterer and feeder (Nelson Products Co., Sioux Rapids, Iowa



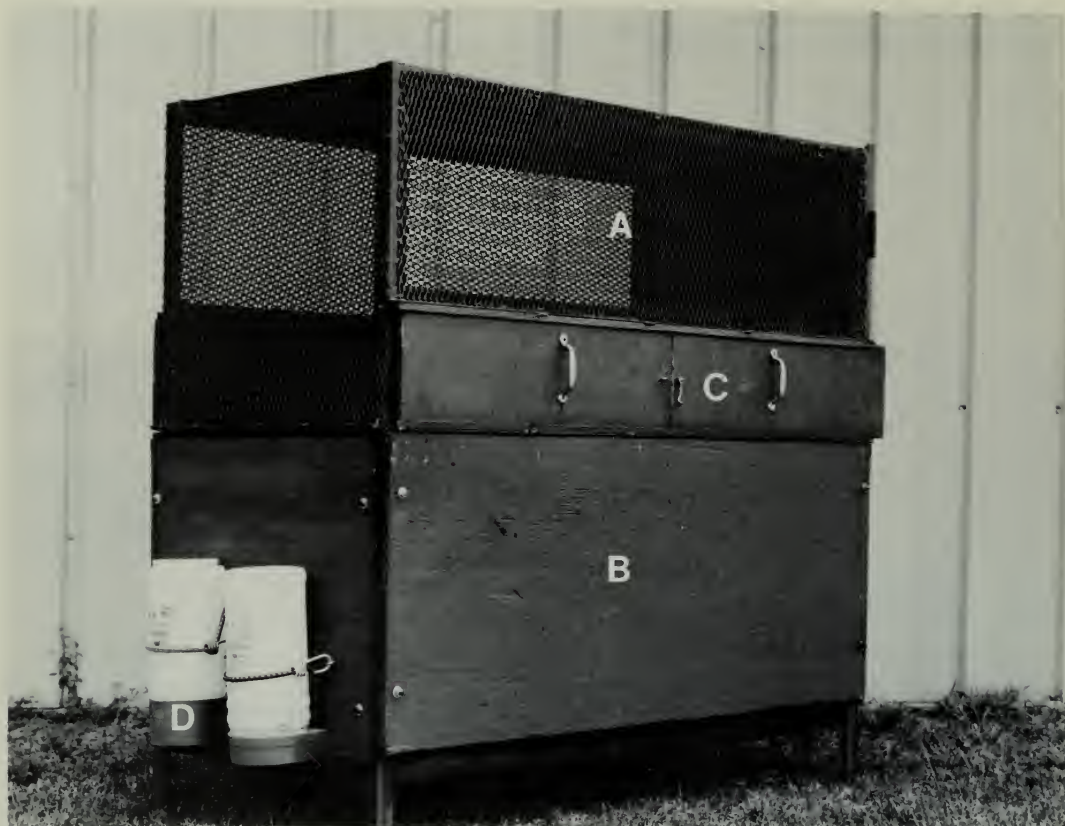


FIGURE 1. Outside view of confinement cage showing general construction features (A–C) and attachment of feeder and waterer (D).

50585). We began by constructing an angle iron frame and welding flattened expanded metal to the sides ( $1.22 \times 0.41$  m), top ( $1.22 \times 0.61$  m), and floor ( $1.22 \times 0.61$  m) (Fig. 1A). Plywood panels were then bolted (shaft of bolts protruding outward for attachment of nuts) to the lower portions of the sides ( $1.22 \times 0.53$  m) and front ( $0.61 \times 0.61$  m) of the frame (Fig. 1B). All expanded metal, plywood, and hardware were attached to the outside framework to prevent injury to the animal from any rough or sharp edges. The expanded metal flooring was reinforced with a steel pipe welded in the center of the frame on the underside of the flooring. A rubber mat slightly smaller than the cage floor was inserted to prevent damage to the feet of the animal by the metal flooring yet allowed wastes and ticks to drop to the stall floor below. Channel iron enabled the plywood doors on the sides ( $0.61 \times 0.20$  m) and back ( $1.14 \times 0.56$  m) to slide (Fig. 1C). The side doors allowed easy access to the host during confinement, whereas the large rear

door allowed for placement and removal of the host. The side doors were actually 2 small doors sliding in opposite directions and were latched together in the center with a screen-door hook when not in use (Fig. 1C). All doors were equipped with screen-door pull handles for easy access. The front plywood panel had openings cut in the lower edge to accommodate pet feeding and watering containers that were attached to the outside with elastic cords (Fig. 1D). An opening of 2.54 cm was left between the bottom of the plywood panel sides and the floor to allow daily cleaning of the cage for removal of wastes and ticks not dropping through the floor. After completion, the cage was painted with rust-proofing paint.

These cages were used for a total of 6 and 8 infestation periods of ca. 30 days duration for goats (unpubl. data) and deer (Cooksey et al., 1988), respectively, with *Boophilus annulatus*. With the exception of 1 deer that was severely injured while caged, animals in the studies were

in good condition when removed at the end of the parasitic period. The injured animal was observed to stress easily even in a large confinement area. Use of tame animals is highly recommended because they are easier to work with due to exposure to cleaning and other activities on a daily basis. Burlap covers can be used to block out light and outside activity for less tame or easily excitable hosts. Cages were cleaned by inserting a hose through a side door to wash the wastes and detached ticks to the stall floor for collection. However, when excitable animals were used, the cages were cleaned by forcing a stream of water into the opening between the bottom of the plywood sides and floor.

Accumulation of heat was a major concern with the confined animals because the temperature and humidity in south Texas were often high. However, the cage design allowed sufficient air circulation when placed inside an open air barn, even if the cage was covered with burlap. The cage was large enough to accommodate an adult white-tailed deer buck without antlers and

still allow for grooming and resting activity. The only problem encountered was the occasional crushing of detached ticks on the mat, but this problem could be remedied by use of rubber-coated expanded metal flooring rather than the rubber mat used in these studies. This cage design can be adapted for use with similar hosts and/or other ectoparasites.

This paper reports the results of research only. Mention of a commercial or a proprietary product in this paper does not constitute an endorsement by the U.S. Department of Agriculture.

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## Suppression of Release of Microfilariae *In Vitro* from *Setaria cervi* (Nematoda: Filariodeae) by Chlorpromazine

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**ABSTRACT:** Chlorpromazine (CPZ) caused suppression in release of microfilariae *in vitro* from *Setaria cervi* at  $10^{-5}$  M concentration. The inhibitory effect of CPZ was further stimulated by  $\text{Ca}^{2+}$  (2 mM), whereas addition of EGTA (2 mM) resulted in partial reversal. Addition of dopamine (1 mM) along with CPZ ( $10^{-5}$  M) caused similar inhibition as was observed in the presence of CPZ ( $10^{-5}$  M) alone. CPZ did not effect *in vitro* oxygen uptake by worm homogenates up to 0.5 mM concentration. The results indicate the  $\text{Ca}^{2+}$ /calmodulin-dependent anthelmintic action of CPZ on *S. cervi*.

Chlorpromazine (CPZ), trifluoperazine (TFP), and related phenothiazines are used widely as antipsychotic drugs (Kaiser and Selzer, 1981). These compounds have been found to possess anthelmintic (Cavier, 1973; Campbell and Rew, 1986) and antiprotozoal (Scheibel et al., 1987;

Kishore and Shukla, 1988) action, but the mechanism of their antiparasitic action is not understood properly. In higher organisms, the calmodulin (CaM)-dependent signal transduction by  $\text{Ca}^{2+}$  has been identified as a site for phenothiazine action (England, 1986). The role of  $\text{Ca}^{2+}$ /CaM in parasite metabolism, a largely neglected area so far, has the potential of improving further approaches to chemotherapy (Scheibel et al., 1987). CPZ also possesses the property of a dopamine antagonist (Levitzki, 1980), providing an alternative target for action. The presence of a CaM system and  $\text{Ca}^{2+}$ -dependent binding of trifluoperazine to this activator protein has been demonstrated recently in helminths (Masruchia et al., 1986; Hipkiss et al., 1987). The present communication reports the inhibitory action of



TABLE I. *Effect of chlorpromazine (CPZ) on the release of microfilariae from Setaria cervi female in vitro.\**

Concentration of the drug	Time of incubation (min)					
	10	20	30	40	50	60
Control	1,876 ± 770	3,417 ± 1,373	5,829 ± 1,541	9,450 ± 2,747	13,132 ± 3,187	13,500 ± 3,316
CPZ (10 <sup>-5</sup> M)	605 ± 252† ( $<0.05$ )	853 ± 370† ( $<0.05$ )	1,927 ± 726† ( $<0.02$ )	2,275 ± 1,011† ( $<0.02$ )	2,812 ± 1,264† ( $<0.01$ )	3,507 ± 1,674† ( $<0.01$ )
CPZ (10 <sup>-4</sup> M)	Nil	Nil	Nil	Nil	Nil	Nil
CPZ (10 <sup>-3</sup> M)	Nil	Nil	Nil	Nil	Nil	Nil
CPZ (10 <sup>-5</sup> M) + CaCl <sub>2</sub> (2 mM)	Nil	Nil	945 ± 427† ( $<0.005$ )	1,281 ± 579† ( $<0.005$ )	1,372 ± 640† ( $<0.002$ )	1,555 ± 701† ( $<0.002$ )
CPZ (10 <sup>-5</sup> M) + EGTA (2 mM)	1,137 ± 422 ( $<0.5$ )	3,120 ± 1,105 ( $<0.8$ )	4,842 ± 1,722 ( $<0.2$ )	5,005 ± 1,917† ( $<0.1$ )	8,742 ± 2,707† ( $<0.2$ )	10,400 ± 3,217 ( $<0.5$ )
CPZ (10 <sup>-5</sup> M) + Dopamine (1 mM)	667 ± 396† ( $<0.05$ )	976 ± 427† ( $<0.05$ )	1,738 ± 640† ( $<0.02$ )	2,379 ± 1,037† ( $<0.02$ )	3,019 ± 1,250† ( $<0.02$ )	3,397 ± 1,860† ( $<0.01$ )

\* Values given are expressed as number of microfilariae released per worm in glucose-rich KRB medium and are mean ± SD of 3 separate sets of experiments. Triplicate observations were obtained for each set.

† Values significantly different than control. Values given in parentheses are *P* values as compared to the controls. Student's *t*-test was employed and a *P* value  $<0.05$  is considered to be statistically significant.

CPZ on *in vitro* release of microfilariae from the bovine filarial parasite *Setaria cervi* and its correlation with CaM- and/or dopamine-binding sites.

Adult motile *S. cervi* parasites were collected from the peritoneal folds of freshly slaughtered Indian water buffalo (*Bubalus bubalis* Linn.), washed, and suspended in Kreb's Ringer buffer saline (KRB) at 37 C and used immediately for the experiments. Adult female parasites were blotted carefully before weighing and transferred to 25-ml flasks containing 10 ml of KRB saline fortified with 10 mM glucose. Drugs at various concentrations were added to individual flasks and incubated at 37 C in a shaking water bath (65 strokes/min). Aliquots from the media were taken at desired time intervals for the counting of microfilariae under a microscope. Oxygen uptake by cell-free homogenates of *S. cervi* were monitored by Gilson oxygraph using a Clark electrode.

Addition of 10<sup>-3</sup> M CPZ to glucose-rich medium containing *S. cervi* female worms resulted in immediate paralysis of the parasites. The motility of the worms, exposed to CPZ for 10 min, could not be recovered by washing and resuspending them in the drug-free medium. At the level of 10<sup>-4</sup> M concentration of the drug the worms did not show any noticeable change up to about 15 min, the motility decreased gradually thereafter, and the worms were paralyzed completely and irreversibly 60 ± 15 min after the exposure. CPZ did not show any observable effect on worm motility at 10<sup>-5</sup> M concentration.

Adult *S. cervi* female parasites maintained at 37 C in glucose-rich KRB saline continuously release microfilariae in the medium. The maximum release of microfilariae was noticed up to 60 ± 20 min of incubation under control conditions. Exposure of these parasites to 10<sup>-3</sup> and 10<sup>-4</sup> M CPZ completely arrested the release of microfilariae in the medium. Marked inhibition (about 73%) of microfilariae release was noticed even at 10<sup>-5</sup> M CPZ (Table I). The antagonism observed in terms of anthelmintic activity of CPZ, i.e., inhibitory effect on the release of microfilariae, was grossly at the appropriate concentration of CPZ required for CaM antagonism, thus fulfilling one of the criteria correlating inhibition of biological response by CPZ to the antagonism of CaM activity (Roufagalis, 1985).

Microfilariae are formed and stored in the uterine sacs of filarial parasites, and by muscular movements they are released continuously. The molecular and biochemical processes involved in the release are not understood properly, but the Ca<sup>2+</sup>/CaM system may have a role in view of its ubiquitous involvement in contractile, locomotive, and secretory functions (Hidaka and Harthshorne, 1985).

Dopamine-binding sites have been detected in *S. cervi* (Tekwani et al., 1986), but addition of dopamine (1 mM) along with CPZ (10<sup>-5</sup> M) yielded the same effect on the arrest of microfilariae release as did 10<sup>-5</sup> M CPZ alone. Thus, dopamine receptors do not seem to be involved in the anthelmintic action of CPZ.

Phenothiazines have been shown to also act

through impairment of mitochondrial function (Kishore and Shukla, 1988). But CPZ did not affect oxygen uptake by cell-free homogenates of *S. cervi* up to  $5 \times 10^{-4}$  M concentration, whereas a  $10^{-3}$  M concentration yielded about 30% inhibition. Inhibition of energy metabolism and mitochondrial function thus would not appear to be the primary target for inhibitory action of CPZ on microfilariae release.

On the other hand, the effects of the calcium chelator, EGTA, and  $\text{Ca}^{2+}$  on modulating the effect of CPZ on microfilariae release support the calcium dependence of the inhibitory action. Addition of EGTA (2 mM) to CPZ ( $10^{-5}$  M) produced the same number of microfilariae released as would be expected to occur between  $10^{-5}$  M CPZ alone and the control value, i.e., the presence of EGTA could partly reverse the inhibitory action of CPZ. Along the same line, 2 mM calcium chloride and  $10^{-5}$  M CPZ yielded greater inhibitory effects on microfilariae release than  $10^{-5}$  M alone, however, the effect still was less than  $10^{-4}$  M CPZ alone.

Because the CaM effects are manifested after the intracellular level of  $\text{Ca}^{2+}$  is increased but they generally are not manifested in the absence of  $\text{Ca}^{2+}$ , and binding of CPZ to CaM has been shown to be  $\text{Ca}^{2+}$  dependent (England, 1986), the studies reported in this communication support the involvement of a  $\text{Ca}^{2+}$ /CaM system in the antifilarial action of CPZ as judged by the inhibition of release of microfilariae and motility of *S. cervi*. However, further studies with different CaM antagonists are needed to correlate their antiCaM potencies and stereospecificity (Roufagalis, 1985) with their effects on microfilarial release as well as their anthelmintic action. Interference in the release of microfilariae and motility of adult worms also have been implicated in the mode of action of other anthelmintics (Townson et al., 1987) and arrest of microfilariae release *in vitro* reported herein thus may provide a rapid method for screening of anthelmintics.

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## The Growth Hormone-Like Factor Produced by the Tapeworm *Spirometra mansonioides* Specifically Binds Receptors on Cultured Human Lymphocytes

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**ABSTRACT:** Plerocercoid larvae of the tapeworm *Spirometra mansonioides* produce a factor with activities similar to those of growth hormone (GH). Highly selective receptors for GH have been described on cultured human lymphocytes (IM-9 cells) and these cells have been used as a model of binding essentially restricted to human GH (hGH). We compared the displacement of [ $^{125}$ I]hGH by hGH and partially purified plerocercoid growth factor (PGF) in assays using rabbit hepatic membranes and IM-9 cells. PGF displaced [ $^{125}$ I]hGH from both rabbit hepatic membranes and IM-9 cells in a dose-dependent manner ( $r > 0.98$ ). These results show that PGF specifically binds to hGH receptors on human IM-9 cells and suggest the possibility that PGF will have somatotrophic activity in humans.

The plerocercoid stage of the tapeworm *Spirometra mansonioides* has the ability to infect a wide variety of vertebrates from amphibians to mammals, including humans. Plerocercoids of this tapeworm are especially interesting as they produce a substance that has distinct growth hormone (GH)-like actions (see Mueller, 1974, for review).

Interaction of hormones with receptors prepared from tissues of animals or humans has been used as a method to support the prediction of biological activity. Human GH is unique in its ability to specifically bind both lactogenic and somatogenic receptors in a wide variety of vertebrates and expresses activities consistent with receptor binding. Tsushima and Friesen (1973) developed a sensitive radioreceptor assay (RRA) for hGH using liver membranes from late pregnant rabbits. Tsushima et al. (1974) later showed that media in which plerocercoids of *S. mansonioides* had been incubated contained a substance that inhibited the binding of [ $^{125}$ I]hGH to rabbit liver receptors in a manner parallel to the hGH standard. Lesniak et al. (1973) reported that only hGH was effective in competing for [ $^{125}$ I]hGH binding to receptors on human lymphoblastoid cells of the IM-9 line.

Partially purified PGF was obtained by a previously described method (Phares and Booth, 1987). Quantitation of PGF was accomplished by RRA using rabbit liver membrane receptors, [ $^{125}$ I]hGH, and hGH as the standard of comparison. The assay procedure used was the method of Tsushima and Friesen (1973) except that we used an overnight incubation and included 0.1% Triton X-100 in the assay buffer. Four dilutions (1/10, 1/20, 1/30, and 1/40) were assayed in duplicate and the mean amount of active PGF in the preparation was expressed as nanogram equivalents (ng eq) of the hGH standard after correcting for the dilution factors. Although it is not possible to determine the actual mass of PGF in the RRA, a mass equivalent based on its binding activity can be assigned. Using this method of quantitation it was possible to estimate the relative potency of PGF in binding the highly select receptors for hGH. The membrane fraction from 21 g of plerocercoids was separated and solubilized to yield PGF with a total activity of 1,570 ng eq/ml and a specific activity of 565 ng eq/mg of protein. Based on the activity obtained in the rabbit liver RRA, the PGF was diluted in a manner that allowed assignment of doses in ng eq/ml. Nine standard doses of PGF over a range of concentrations (20-400 ng eq/ml) were then reassayed in rabbit liver membrane-binding assay and the binding data were analyzed by logit-log regression and compared to binding curves obtained using hGH as the competitive inhibitor of [ $^{125}$ I]hGH binding. The data in Table I are the results of 3 independent assays of PGF and hGH and they show that there were no statistically significant differences between PGF and hGH.

Standard doses of the same preparation of PGF were then tested for specific binding activity in an IM-9 cell RRA. Cultured human lymphocytes of the IM-9 cell line were obtained from the Human Genetic Mutant Cell Repository Institute

TABLE I. Comparison of receptor binding of hGH and PGF.\*

ED <sub>50</sub> †	Binding potency‡ (%)	Minimal detectable dose	Slope§	r
Source of receptors: hepatic membranes from a pregnant rabbit				
hGH 95 ± 10 ng/ml	100%	5 ± 2 ng/ml	-1.01 ± 0.04	0.99 ± 0.01
PGF 103 ± 7 ng eq/ml	92%	7 ± 2 ng eq/ml	-0.97 ± 0.11	0.97 ± 0.01
Source of receptors: human IM-9 cells				
hGH 129 ± 18 ng/ml	100%	10 ± 4 ng/ml	-1.02 ± 0.03	0.99 ± 0.01
PGF 295 ± 59# ng eq/ml	45%	26 ± 6# ng eq/ml	-1.40 ± 0.24	0.99 ± 0.01

\* Results are the mean ± SE of 3 separate experiments for both sources of receptors. Mean specific binding of [<sup>125</sup>I]hGH in the assays using rabbit liver membranes was 38 ± 2% and 15.2 ± 0.7% using IM-9 cells.

† ED<sub>50</sub> was calculated from the logit-log curve and was the dose of hGH or PGF required to displace 50% of the [<sup>125</sup>I]hGH.

‡ Calculated by ED<sub>50</sub> for hGH/ED<sub>50</sub> for PGF × 100.

§ Slopes of logit-log curves.

|| r = correlation coefficient of logit-log curves.

# P < 0.05.

for Medical Research (Camden, New Jersey). To each of duplicate assay tubes was added 12 × 10<sup>6</sup> viable IM-9 cells, approximately 50,000 cpm of [<sup>125</sup>I]hGH, and increasing doses of hGH or PGF, and the RRA was conducted as described (Lesniak and Roth, 1977) except 0.02% Triton X-100 was included in the buffer. Three separate RRA's using IM-9 cells were conducted and binding curves for hGH and PGF were analyzed independently by logit-log regression. When assayed as an unknown competitive inhibitor of [<sup>125</sup>I]hGH binding, the mean binding activity of PGF was 625 ng eq/ml. Although it is obvious that PGF specifically binds hGH receptor on IM-9 cells, it did so with less potency compared to its binding activity in the rabbit liver RRA. PGF displaced [<sup>125</sup>I]hGH from its receptors on IM-9 cells in a dose-dependent manner, and the displacement curves produced by PGF or hGH were similar. The data from the logit-log transformed binding data are presented in Table I and substantiate that PGF has specific hGH-like activity in binding IM-9 cells. The mean ED<sub>50</sub> of hGH in 3 IM-9 cell RRA's was 129 ng/ml compared to a mean ED<sub>50</sub> for PGF of 290 ng eq/ml, suggesting a binding potency in the human cell line for PGF equal to 45% that of hGH.

These results are in conflict with those of Carr and Friesen (1976) who found essentially no binding of PGF in human liver slices. As no details of the experimental design or data involved in testing PGF in human liver slices were given by the authors, their conclusion is difficult to evaluate. The facts that their preparation of PGF contained only 150 ng eq/ml (vs. 1,570 ng eq/ml in the current study) and that they included no detergent in their binding assay may have

contributed to the absence of detectable binding for PGF in human liver slices.

The GH receptors on cultured human lymphocytes, like those in human liver, are extremely hormone and species specific as they show virtually no cross-reactivity with nonprimate GH's and do not bind any prolactin, including human prolactin. The difference in the potency of PGF in the rabbit liver and IM-9 cell assays of the current studies suggests that PGF is similar but not identical to hGH with respect to its receptor-binding characteristics.

In any event, the data show that the GH-like factor from plerocercoids of *S. mansonioides* binds to the highly selective receptors on human IM-9 cells but with less affinity than it does to receptors in rabbit membranes. These results suggest the possibility that PGF will have GH-like activities in humans.

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## Subclinical Cryptosporidiosis of Turkeys in Iowa

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**ABSTRACT:** *Cryptosporidium* oocysts were found in the feces of 16 of 20 (80%) 17-day-old and 38 of 100 (38%) 24-day-old turkeys in a healthy commercial flock in central Iowa. Mean dimensions of 50 oocysts were  $4.5 \times 5.3 \mu\text{m}$ . In experimentally inoculated turkeys and chickens, the parasites frequently infected the ceca, colon, and cloaca, but rarely infected the small intestine or bursa of Fabricius. These data differ from published descriptions of *Cryptosporidium baileyi* in chickens and turkeys. The results indicate that the cryptosporidia of poultry are not a homogeneous group and demonstrate that *Cryptosporidium* infections can occur in apparently healthy poultry flocks.

Coccidian parasites of the genus *Cryptosporidium* (Apicomplexa, Coccidiasina) are pathogens of birds, mammals, and other hosts (Fayer and Ungar, 1986). Both respiratory and intestinal infections have been described in poultry, sometimes in association with severe disease and death (Slavin, 1955; Hoerr et al., 1978, 1986; Dhillon et al., 1981), but there are no clear data on the prevalence of such infections. The taxonomy of the cryptosporidia of poultry is also unclear. Only 1 species, *C. baileyi*, has been thoroughly characterized (Current et al., 1986; Lindsay et al., 1986a, 1986b, 1987). The validity of the remaining named species, *C. meleagridis* Slavin, 1955, *C. tyzzeri* Levine, 1961, and *C. anserinum* Proctor and Kemp, 1974, is uncertain. A recent morphometric study of oocysts from turkey feces revealed a population of oocysts significantly smaller and more spherical than those of *C. baileyi*, and suggested that *C. meleagridis* may be a valid designation for small cryptosporidial oocysts from turkeys (Lindsay, D. S., B. L. Blagburn, and C. A. Sundermann, 1987, 62nd Annual Meeting, American Society of Parasitologists, Abstract 107).

We collected fecal samples from pans beneath cages of clinically normal birds in a commercial turkey-raising facility in central Iowa. The facility had no history of severe intestinal or respiratory disease in its birds. Samples were examined for *Cryptosporidium* oocysts using Sheather's sucrose flotation (Sloss and Kemp, 1978). The prevalence of oocysts in samples from turkeys of different ages was as follows: 5-day-old, 0 of 20; 17-day-old, 16 of 20 (80%); 24-day-old, 38 of 100 (38%); 60-day-old, 0 of 10; 9-wk-old, 0 of 10. Oocysts were oval to spherical and contained 4 sporozoites and a residuum. The oocyst wall was smooth, thin, and colorless; no plugs or sutures were visible with the light microscope. Fifty oocysts in Sheather's sucrose solution were measured with an ocular micrometer and had mean dimensions of  $5.3 (\text{SD} = \pm 0.2) \times 4.5 (\pm 0.2) \mu\text{m}$ ; the mean shape index was  $1.18 (\pm 0.07)$ .

Samples containing oocysts were pooled and oocysts were isolated by coverslip flotation in Sheather's sucrose solution, washed 3 times in Dulbecco's phosphate-buffered saline, and inoculated into 2 6-wk-old chickens ( $2.5 \times 10^3$  oocysts/bird). Two age-matched control chickens were housed in a separate cage. Feces were collected in pans containing 2.5% potassium dichromate placed beneath the cages. One principal and 1 control bird were killed 7 and 11 days postinoculation (PI). Samples of esophagus, proventriculus, duodenum, upper, mid, and lower small intestine (10 cm posterior from the pylorus, 40 cm anterior to the ceca, and 10 cm anterior to the ceca, respectively), ceca, colon, bursa of Fabricius, cloaca, and trachea were fixed in 10% buffered formalin for histologic examination by standard techniques. Oocysts were iso-

TABLE 1. Distribution of cryptosporidia in experimentally infected chickens and turkeys.

	Chickens		Turkeys			
	#1* (7†)	#2 (11)	#1 (5)	#2 (5)	#3 (7)	#4 (7)
Esophagus	-	-	-	-	-	-
Proventriculus	-	-	-	-	-	-
Gizzard	-	-	-	-	-	-
Duodenum	-	-	-	-	-	-
Upper small intestine	-	-	-	-	-	-
Mid small intestine	-	-	-	-	-	-
Lower small intestine	-	-	+	-	-	-
Cecum	+	-	+	+	+	+
Colon	+	-	+	+	+	+
Bursa	-	-	-	-	+	-
Cloaca	+	+	ND‡	ND	ND	ND
Trachea	-	-	-	-	-	-

\* Identification number.

† Days PI at necropsy.

‡ Not examined.

lated from the chicken feces by coverslip flotation and inoculated into 4 11-day-old turkey poults ( $3.0 \times 10^5$  oocysts/bird). Two similar poults, housed in a separate cage, served as controls. Fecal samples were collected from pans beneath cages daily and examined for oocysts by Sheather's sucrose flotation. Two principals and 1 control bird were killed 5 and 7 days PI and histologic samples were taken as before.

Chickens inoculated with oocysts from turkey feces became infected and passed oocysts that were infectious for turkeys. Oocysts were demonstrated from 4 to 7 days PI and from 2 to 6 days PI in the feces of the experimentally infected chickens and turkeys, respectively, but the small number of birds in the experiments precluded accurate determination of the prepatent or patent periods. No attempt was made to determine the abundance of oocysts in the fecal samples but we were unable to recover more than  $2 \times 10^6$  oocysts from the feces collected during the transmission experiments. Our inability to recover large numbers of oocysts limited the number of birds that could be used in the transmission experiments and discouraged us from attempting further analysis of the isolate.

Histologic examination of the samples taken from experimentally infected birds showed that parasites developed in the lower intestinal tract, particularly the ceca, colon, and cloaca (Table I). Distribution of parasites within infected tissues was uneven: the number of parasites per  $40\times$  microscopic field ranged from 0 to  $>50$  within individual histologic sections. Intensity of infection appeared highest in cecum and cloaca and lowest in bursa and small intestine. No histologic

lesions were observed except displacement of microvilli at the point of parasite attachment and none of the birds became clinically ill. Neither oocysts in feces nor parasites in histologic sections were observed in control birds.

The *Cryptosporidium* isolate observed in the present study appeared to differ from *C. baileyi* in 2 respects. First, oocysts were smaller and more spherical than those of *C. baileyi* for which oocyst dimensions of  $6.2 \times 4.6 \mu\text{m}$  (Current et al., 1986) and  $6.8 \times 5.0 \mu\text{m}$  (Lindsay et al., 1986b) have been reported. They conformed precisely, however, to a population of oocysts with mean dimensions of  $5.2 \times 4.6 \mu\text{m}$  recently described from turkey feces and interpreted to be oocysts of *C. meleagridis* (Lindsay et al., 1987, loc. cit.). Second, our isolate consistently infected the ceca but failed to consistently infect the bursa of Fabricius of orally inoculated birds. This pattern is the opposite of that found in studies of *C. baileyi* tissue distributions in chickens and turkeys (Current et al., 1986; Lindsay et al., 1986b, 1987).

It is not possible for us to conclude whether the parasites we observed belong to a species distinct from *C. baileyi* or are examples of variability within the species. Our data show, however, that the cryptosporidia of poultry are not a homogeneous group and that additional research is required to determine the diversity of these organisms and to establish useful criteria for the designation of species. The fact that subclinical cryptosporidiosis could be so easily demonstrated in an arbitrarily chosen flock raises the possibility that it is a common condition in poultry facilities. The epidemiology of subclinical cryptosporidiosis and the possibility that it has



an economically significant impact on poultry production should be examined.

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## The Spiny Rat Louse, *Polyplax spinulosa*, as a Parasite of the Rice Rat, *Oryzomys palustris*, in North America

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**ABSTRACT:** The spiny rat louse, *Polyplax spinulosa*, was collected, as adults and embryonated ova from the rice rat, *Oryzomys palustris*, a cricetid rodent, in Davidson Co., Tennessee. This sucking louse is typically parasitic on domestic rats, which are murid rodents. Because most sucking lice are normally host specific, such cross-familial host infestation is noteworthy.

*Hoplopleura oryomydis* Pratt and Lane is the normal sucking louse associate of the rice rat, *Oryzomys palustris* (Harlan), in North America (Pratt and Lane, 1951; Kim et al., 1986). Five adult lice and a sample of louse ova (nits) retrieved by the author from an adult male *O. palustris* live-trapped near Nashville, Davidson Co., Tennessee, on 29 April 1978 were therefore assumed to belong to this species. However, when these lice were cleared (in 10% KOH), stained (with orange G), and slide-mounted, all 5 specimens (4 females, 1 male) were identified as the spiny rat louse, *Polyplax spinulosa* (Burmeister), a common parasite of domestic rats throughout

much of the world. Nit morphology also conforms to that described for *P. spinulosa* by Pratt and Karp (1953). Voucher specimens have been deposited in the U.S. National Museum under accession no. ENT-A-3305.

The host specimen was not retained but the hairs onto which the nits were cemented very clearly belong to *O. palustris* (see Moore and Braun, 1983), thus confirming the original host identification.

This appears to be the first record of *P. spinulosa* parasitizing *O. palustris*; neither Hopkins (1949) nor Kim et al. (1986) list the spiny rat louse from the rice rat. Almost certainly, this infestation was accidental. The rice rat host was trapped on a muddy bank along a small tributary of the Cumberland River close to storage buildings that were occupied by domestic rats (and house mice). Interspecific contacts between rice rats and domestic rats could have facilitated louse exchanges between them. Such exchanges are un-

usual because most sucking lice are host specific (Kim, 1985), and because domestic rats are murid rodents but rice rats are cricetids. Interestingly, the presence of embryonated *P. spinulosa* nits on the rice rat suggests that the lice were more than stragglers and may have been reproducing, although no immatures were collected to corroborate this. It would be of interest to ascertain whether *P. spinulosa* can transmit pathogenic microorganisms such as *Rickettsia typhi* and *Haemobartonella muris* to rice rats as it can between domestic rats (Kim et al., 1973, 1986).

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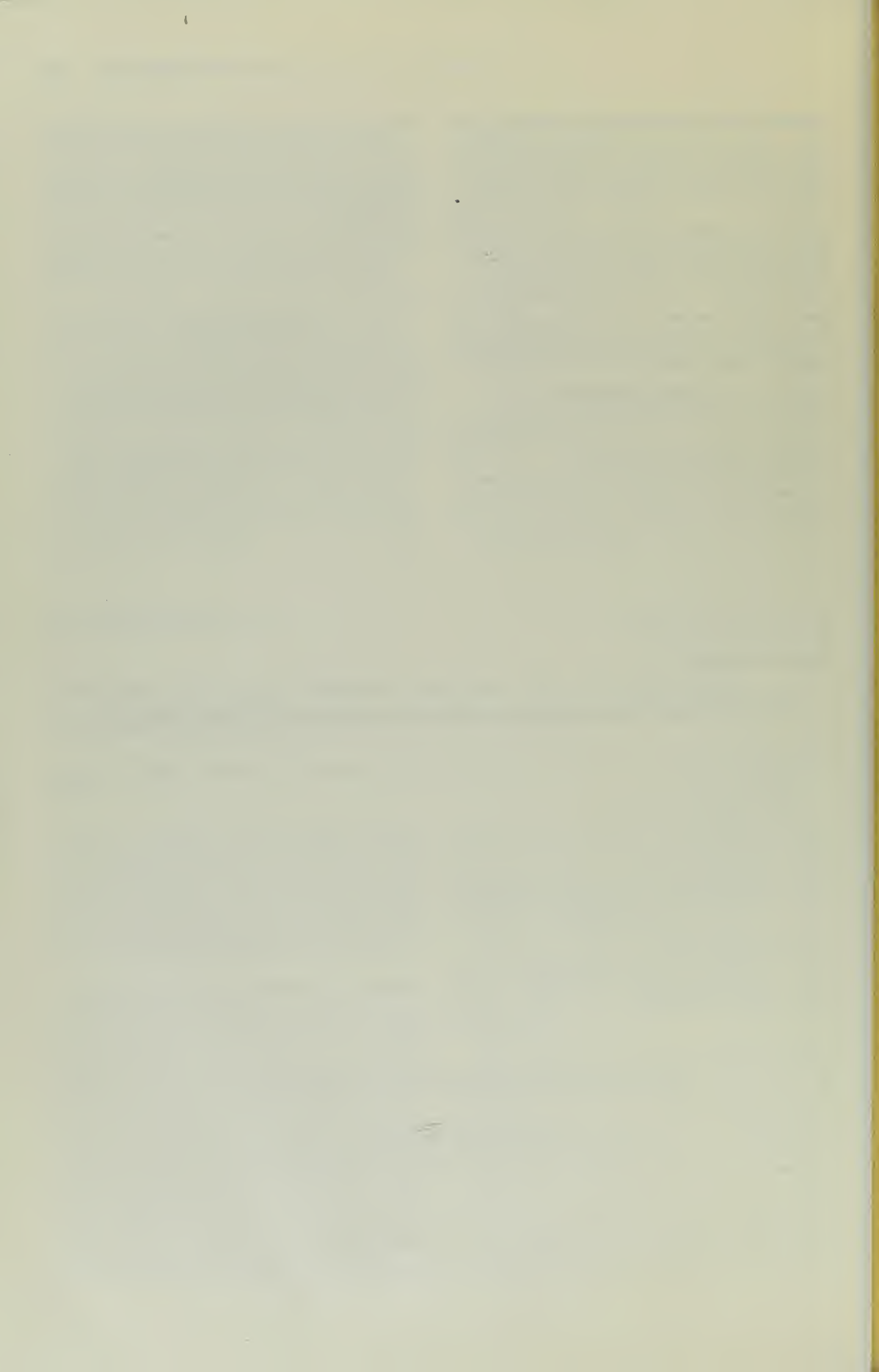
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## **EDITOR'S FAREWELL**

With this issue of the *Journal*, our job as Editors ends. We would like to thank all members of the Society who have made our task easier and more enjoyable during the past 5 years. The editorial job afforded us the opportunity to become better acquainted with many of you and your interesting research in what has been an enriching experience.

We wish our new Editor, Brent Nickol, the best of luck and are confident that you will offer him the same generous support and encouragement given us to maintain the high standards of our *Journal*.

**Sherwin S. Desser and David F. Mettrick**





*Gilbert A. Castro*

## PRESIDENTIAL ADDRESS\*

### Gilbert A. Castro

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#### Introduction of President Castro, by Donald W. Duszynski

Fellow members of the American Society of Parasitologists, friends, and distinguished guests, I welcome you this morning to listen to the President of our Society deliver his Presidential Address, entitled "Pariahs of the Scientific Caste System." Webster defines pariah as "an outcast." I tell you this because it is customary for introductions on these solemn occasions to be given by a former student, a close professional colleague, or an old buddy from graduate school; I am none of these, but given the title of Gilbert's talk, I think I know why he selected me to introduce him: I tried to work in his laboratory 1 semester and we spent a lifetime fighting with each other. When Dr. Castro asked me to make this introduction, he asked me to "be friendly." He promised not to ramble and to be succinct in his talk. We'll see if either of us can live up to such reasonable expectations.

Gilbert Anthony Castro was born 24 April 1939 in Port Arthur, Texas, 1 of 7 children to Richard N. and Pauline Castro. Neither of his parents, who still live in Port Arthur, completed high school, but they apparently instilled in their children the need and the value of education. All siblings completed high school, 5 have college diplomas, 1 is working toward a master's degree, and 2 have Ph.D.'s and postdoctoral training. But Gilbert's path in this direction was not initially obvious; he graduated scholastically in the lower half of his high school class of 465 students. Although he did not excel in the classroom in his early years, certain aspects of his personality were always keen, especially his desire to win. He lettered in baseball and basketball in junior high and high school, and his high school basketball team won the Texas 4A State Championship in 1957, when Gilbert was a junior. Gilbert was the "sixth starter" on that team, and "basketball star Castro" (as the papers described him) worked as a service station attendant, a manual laborer for the city, and a tugboat deckhand and cook for Wardlaw Towing Company, Port Arthur. Manual labor in those early years probably convinced Gilbert not to repeat the sterling academic performance he had given in high school because he graduated from Lamar Tech in August 1961 with a B.S. in Biology and, upon graduation, was awarded a 3-year Graduate Scholarship for \$6,600 to attend the University of Arkansas. After completing the M.S. in Zoology at Arkansas in 1963, Gilbert went to work with Professor Leroy Olson at The University of Texas Medical Branch, Galveston, where he earned the Ph.D. in Microbiology in 1966. He then spent 2 years doing postdoctoral work with Dr. Donald Fairburn at the University of Massachusetts. In 1968 he accepted his first position, an assistant professorship, in the School of Public Health at the University of Oklahoma Health Science Center, Oklahoma City. Within 4 years—a very short time, even then—he had progressed to associate professor with tenure, and in 1972 he was recruited by the Department of Physiology, The University of Texas Medical School, Houston, where he works today as Professor of Physiology and Cell Biology and where he has been a major force in the development and growth of that medical school for the last 16 years. The rest, as they say, is history.

Dr. Castro is an internationally recognized scholar and authority on the interactions between gastrointestinal parasites and their hosts. The hallmark of his approach is the application of biochemical and physiological concepts and techniques to identify the mechanisms of host-parasite interactions at the cell and molecular biological levels. His research subjects are mostly helminths in lab animals, especially *Trichinella spiralis*, although I did get him once to take off his blinders and work with the Coccidia during the 5 months I spent in his lab in 1976. Before I went to Houston, I first came to know Gilbert personally through his presentations of papers at regional and national parasitology meetings. Unless you have heard him speak and seen his mind at work, you can't appreciate the impact and impression he makes on his audience. Paraphrasing written comments from his professional colleagues may give you a sense of the esteem his work has received (and I quote from various confidential sources): "His presentations are flawless, his ideas are original and he has great vision; his science is forthright and thorough, the statistical analyses of his data are meticulous, and he interprets his results in the light of a conceptual framework with unusual insight and honesty. His research accomplishments have truly opened new frontiers impacting on a variety of disciplines." After hearing Gilbert speak at a national meeting several years ago, Jack Bristol commented to me, "Once Castro finishes a study, it's ready to put into textbooks." In fact, much of his stuff does go into textbooks because he writes the chapters; over 20 invited book chapters and review articles supplement his 60 original journal articles.

In addition to his science, other professional accomplishments of the Leader of our Society are equally impressive: he has presented numerous papers regionally, nationally, and worldwide, often as an invited speaker; he was the recipient of a prestigious Research Career Development Award from the National Institutes of Health; he has been a consultant for the American Medical Association's Education Project in Southeast Asia; he has served as a Visiting Lecturer in Parasitology, or as a Visiting Professor, at the University of Saigon Medical School, South Vietnam, at the American University of Beirut, Lebanon, at the Second Medical College, Beijing,

\* Presidential Address, 63rd Annual Meeting, American Society of Parasitologists, 3 August 1988, Winston-Salem, North Carolina.



People's Republic of China, and at McMaster University, Hamilton, Ontario, Canada; he has had nearly continuous NIH funding since receiving his first RO1 award in 1970; he has served on numerous national and international review panels, study sections, and committees; he has sponsored a dozen degree candidates and postdoctoral associates; he has served ASP as our Vice President (1983–1984), as an elected member of Council (1979–1982), on many committees—usually as chairman, on the Editorial Board of the Journal (1980–1989), and in 1977 our Society awarded him the Henry Baldwin Ward Medal. There are many other achievements I could cite, but the lesson on Gilbert's CV is over. The hallmark of these and unmentioned accomplishments is the quality of Dr. Castro's performance in the academic arena; this can be assessed by the world class journals where his work has been published (*Proceedings of the Society of Experimental Biology*, *Gastroenterology*, *Annals of Surgery*, *American Journal of Physiology*, *Surgery Research*, and others, in addition to the *Journal of Parasitology*) and from the receipt of the various awards, honors, and lectureships related to his research activities. These creative activities introduce you to the Gilbert Castro many of you know, Castro the Scientist. But Gilbert is not a unidimensional personality.

He is also Castro the Fisherman. Fishing has always been a passion with Gilbert, and as a child he was known all along the Gulf Coast for his exploits as a fisherman. When asked the secret of his success of always catching the most and the biggest fish when others with technologically superior gear usually failed, young Castro replied (and I quote), “##@\$~&\*##@.” Most anglers, too embarrassed to admit they didn't understand him, usually let the question beg. One day when fishing with Gilbert I asked the usual question after he had just caught a large fish and I got the same response, “##@\$~&\*##@.” Undaunted by my apparent ignorance at his colloquial secret I persisted. “Gilbert, tell me in English, how do you catch so many fish?” With that look of disgust on his face that only Castro can give (and many of you have seen it), he glared at me, then spat the wad from his mouth and scorned, “Donald, you've got to keep your worms warm!” So fishing and Gilbert's interest in worm physiology coevolved early in his life.

There are still other sides of Dr. Castro that you should know about. There is Castro the Cisco Kid of Hunting, Castro the Watermelon Seed Spitting Champion of Houston, and Castro the “Pariah” of Social Drinkers. There are stories about each of these, but unfortunately, I don't have time to pursue them.

Finally, there is Castro the Devoted Father to his 2 lovely daughters, Mitzi and Theresa. And Castro the Loving and Dedicated Husband who with his beautiful wife, Georgia Faye, celebrated their 25th wedding anniversary in December 1986.

Ladies and gentlemen, it is with a great deal of personal pleasure that I can introduce to you, the President of the American Society of Parasitologists, Gilbert Anthony Castro. But, before he begins his Address I have just one question: Why is this man always smiling?

### PARIAHS OF THE SCIENTIFIC CASTE SYSTEM

Who are the Pariahs of the scientific caste system? No doubt many of you in the audience know the answer to this riddle, since the subject is a phrase borrowed from an article by one of our society's most distinguished former members, Justus F. Mueller. A copy of his article, entitled *From Rags to Riches, or, The Perils of a Parasitologist* (Mueller, 1961), was given to me as a graduate student by my mentor, Leroy Olson. I surmised that Dr. Olson was trying to warn me of potential pitfalls associated with the field of specialization that I chose to enter. Whatever his reason, I vividly remember Mueller's message.

Justus Mueller presents in his article a most insightful and unforgettable glimpse of life in the field of parasitology. He describes in his uniquely humorous way a feast or famine world and alludes to misunderstandings and misconceptions that have placed parasitologists in a less than enviable position in the scientific hierarchy. His awareness of a scientific peck order became acute when he completed his graduate studies in 1928

and embarked on his own career. Mueller's description of ranking among scientific professions is worth quoting:

At the head of the procession came the experimental zoologists, who specialized in sticking a glass needle into a frog's egg, or shaking echinoderm ova in butyric acid. Next came the geneticists with their fruit flies. After them in order, the cytologists, the embryologists, the animal behaviorists, the comparative anatomists, the invertebrate zoologists, the ecologists, the taxonomists, and last, indistinguishable from camp followers, the parasitologists.

One of Mueller's colleagues reported to him that an experimental zoologist, whom they both knew, stated that parasitology was the absolute nadir of science, from which it was impossible to go lower. Although this statement must have been difficult for a young parasitologist in a new job to swallow, it was evidently apparent to Mueller why the zoologist arrived at his conclusion. Mueller noted, with what must have been some degree of despair, but with message-bearing humor, that:

For as is so often the case among the poor and outcast, those who by all logic should have held together

# RF Illustrated

## TROPICAL MEDICINE REVISITED

After an absence of 25 years, The Rockefeller Foundation is launching a major, worldwide, cooperative effort. Why?

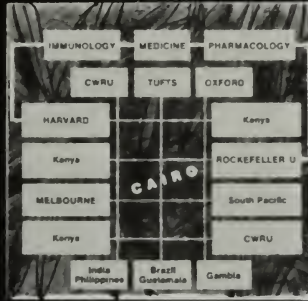


FIGURE 1. Excerpts from article by Warren and Romney (1978) in *Rockefeller Illustrated* announcing plans for the assault on the great neglected diseases of mankind.

in the face of the common enemy, instead discriminated against each other. The blood parasitologists regarded themselves as distinctly superior to those who worked on the fauna of the intestine. These latter just had no status at all. They were in fact the *Pariahs of the scientific caste system*.

Indeed, parasitology is a perilous science. The perils exist within as well as outside the discipline. Clearly, parasitology has its ups and downs. Mueller pointed out that World Wars I and II lifted parasitology from rags to riches because the diseases that parasitologists studied were no longer considered with passing curiosity. Diseases formerly viewed as exotic were presenting real problems on the global scene and required immediate attention. During the past decade medical parasitology gained global attention once again. This was in large part due not to a world war, but to the war on the great neglected diseases of mankind. That effort was spearheaded by the Rockefeller Foundation under the directorship of Kenneth Warren (Warren and Romney, 1978; Fig. 1), our keynote speaker for this meeting. During that period the blood parasitologists at-

tained prominence, if not dominance, in the field. They had the good fortune of going from "rags to riches." Like Justus Mueller, however, I believe in the scripture "the first will be last and the last first." I predict that the next decade will be a most exciting time for those who work on the fauna of the intestine—it should be a decade in which studies of enteric parasitism flourish.

My prediction is based on several recent developments. First and foremost, the gastrointestinal (GI) tract is where students of the fields of endocrinology, neurobiology, and immunology are entering new territories in integrative biology in an effort to understand relationships of structure and function and the regulation of those relationships from systems to the molecular level. Recent developments in these areas were discussed at this meeting in the President's Symposium (Fig. 2).

My prediction that the Pariahs will have their day in the sun is also dependent on those in related fields recognizing the value of employing parasites to probe almost every aspect of gut function. I envision parasites as contributing sig-





## President's Symposium 1988

## The Gut Environment In The Parasitized Host

August 1, 1988 10:30 AM  
Brendle Auditorium  
Wake Forest University

A. Dean Befus, Ph.D.  
Microbiology and Infectious Diseases  
The University of Calgary  
Calgary, Alberta

Immunological Elements  
of the GI Tract: Potential  
Modes of Interaction with  
Physiological Systems

Jack Wood, Ph.D.  
Department of Physiology  
Ohio State Medical School  
Columbus, Ohio

Enteric Nerve Network: The  
"Little Brain" that  
Coordinates Gut Function

William Weems, Ph.D.  
Department of Physiology  
and Cell Biology  
University of Texas Medical School  
Houston, Texas

Intestinal Fluid-Propelling  
Behavior: Its Production,  
Control and Response to  
Perturbations

Don W. Powell, M.D.  
Department of Medicine  
University of North Carolina  
Chapel Hill, North Carolina

Epithelium: Regulation of  
Function by Mediators of  
Inflammation

Habitation of the gastrointestinal (GI) tract by parasites and the local changes in structure and function which they cause are connoted in the phrase "host-parasite relationship". Parasite-induced changes can be viewed from the standpoint of how the GI tract responds to complex injury or how a parasite adjusts to environmental alterations. The byword in both cases is adaptation – the fitness of an organism for its environment or the process by which it becomes fit. Physiologically, adaptation means a process of adjustment to an environmental stress in order to escape from that stress. Biologically, the ultimate function would be to maintain maximum reproductive capacity. Thus, adaptations are perceived to serve useful functions. The objective is to consider changes in GI physiology that occur in the infected host, with emphasis on physiological and immunological interactions, i.e. immunophysiology. The speakers hope to impart the message that changes in smooth muscle and epithelial physiology of the GI tract can be modulated by the mucosal immune system through paracrine, endocrine and neural pathways. Based on knowledge of integrated control systems, an observer can better interpret the role of such changes relative to the pathogenesis of disease and to adaptation.

FIGURE 2. An immunophysiological view of the gut as an environment for parasites.

nificantly to an integrative understanding of the GI tract. Intestinal parasites will become more widely studied by those in related disciplines, not only for public health and economic reasons, but because of a growing recognition that parasites are valuable research "tools." Predictable developmental patterns and the ability to quantify their numbers within host tissues make parasites

suitable as controlled, biological stimuli to perturb physiological functions. Site specificity of parasites raises intriguing research possibilities. Parasites that live free in the gut lumen, that are confined to enterocytes, or that invade subepithelial tissues can be employed to determine how an injurious stimulus applied at one point affects functions at other levels within the gut wall. In-

duced changes can be monitored individually or in concert (Castro, 1976, 1981). Without doubt host-parasite systems offer unique opportunities for investigations of GI physiology, pathophysiology, and immunopathology.

To ensure that those opportunities are used advantageously, we must teach what parasitology can do for other fields. As a play on the words of David Lincicome (1971), we must teach "the goodness of parasitism." Clearly part of our purpose as a society is to promote parasitology through teaching. Regardless of our specific scientific interests, we can all participate in that effort.

As Donald Duszynski, our program chairman, can attest the topic that I chose for this address is not the one originally submitted. I switched to the current topic, which can be directed toward the teaching mission of our society, following a long-distance telephone conversation with a friend and respected scientific colleague (a non-member of ASP). That individual relayed to me the view that enteric parasites and parasite-induced changes in the gastrointestinal tract represent esoteric organisms and esoteric responses, respectively. The context of our spirited discussion is not important other than to say that I was a bit miffed, not with my colleague, but by the view that he conveyed—a view that I have heard expressed many times, a view based on misunderstanding and misconceptions. Individuals that hold this view do not fully understand our field. Therefore we must teach them what parasitology encompasses.

An important aspect of our annual meeting relates to what we can learn from one another. At the very least we can try to understand each other's work so that we can enthusiastically teach about our discipline as a whole. Therefore, I have seized the opportunity presented by this occasion to illustrate my point that studies of enteric parasites and the responses they induce are not esoteric scientific endeavors. To describe enteric parasitism as esoteric is to infer that it is private, secret, confidential, or understood by only a select few. It implies a body of knowledge that is meant only for a specially initiated group. I will demonstrate that this is not the case. Furthermore, I will demonstrate how parasitological concepts and enteric host-parasite systems can be used to advantage by those in related fields to answer biological questions in those fields.

Having been associated with medical schools

for the past 25 years, I have witnessed a considerable compression of time in the curriculum for the teaching of parasitology. Accordingly I have reduced my teaching of the subject to medical students into essentials, which I refer to as the ABC's of medical parasitology.

The ABC's represent 3 questions asked about the host-parasite relationship and, as such, require answers that involve input from the numerous subdisciplines that make up parasitology. The 3 questions which are pertinent to physicians are: Where and how does a person become infected? How is it determined that a person is infected? What can be done to terminate the infection, alter its course, or prevent reinfection? These questions, or rather variations of them, were first presented to me for consideration by my former teacher, Haig Najarian, who is presently Professor of Biology at the University of Southern Maine. Since these questions were unusually helpful to me in learning about parasites, I have used them as the superstructure to build an approach toward teaching about parasites. In an attempt to make the following comments as cohesive as possible I will address those 3 questions using *Trichinella spiralis* and trichinosis as the prototype parasite and parasitic disease, respectively.

Question A: Where and how does a host become infected? To answer this question requires knowledge of the geographic distribution of host species and an understanding of parasite life cycles. This is an ominous task to be sure, but one that can be summarized in bite-size portions to fit the time available or the occasion.

A parasite that I have worked with for some years is *T. spiralis*. All stages in its life cycle inhabit a single host. The parasite occurs in polar, temperate, and tropical regions around the globe and has one of widest host ranges of any nematode which parasitizes humans. Infection is acquired by ingesting the muscle-stage larvae in infected meat.

Question B: How is it determined that a host is infected? In general the definitive answer to this question depends on recovering some stage of the parasite from host tissues, excretions, or secretions. This feat also requires knowledge of the parasite's life cycle. In the case of trichinosis a definitive diagnosis can be made by recovering L<sub>1</sub> stages in skeletal muscle. Presumption of infection may be based on the development of signs and symptoms in the host caused by the parasite



as it develops and reproduces. At this point one ventures into the realm of host-parasite interactions.

To illustrate the immunophysiological and immunopathological bases for symptoms that impact on a presumptive diagnosis, consideration is given to the intestinal phase of trichinosis as summarized from responses in a number of host species (Castro, 1976; Fig. 3). The dynamics of enteric parasitism must be envisioned as a kaleidoscope of physiological events initiated when the parasite enters the GI tract and modulated by its subsequent growth and development. Immunological and physiological vestiges of the interaction may be evident long after the parasite leaves the intestine (Castro and Harari, 1982). The pivotal component in the host-parasite interaction, as presented in Figure 3, is inflammation. This reaction underlies changes in epithelial and smooth muscle effector responses (Castro, 1982; Palmer and Castro, 1986; Alizadeh et al., 1987). Inflammation may be induced nonspecifically or specifically, depending on the stimulus involved. The stimulus for nonspecific induction may be mechanical, chemical, or enzymatic in nature. Antigens provide specific stimulation through immunological pathways. Immune-mediated inflammation can best be appreciated by considering local hypersensitivity responses. These can be categorized into 4 types. Type I is an anaphylactic reaction involving homocytotropic antibodies and amine containing cells such as mast cells, Type II is represented by antibody-dependent cellular cytotoxicity, Type III is represented by the Arthus reaction, and Type IV is represented by the classical delayed hypersensitivity response (Coombs and Gell, 1975).

The changes in physiology induced by inflammation can be viewed in 2 ways, from the standpoint of the pathogenesis of disease and from the standpoint of adaptation. The latter is based on the assumption that physiological changes or adjustments allow the host to cope better with alterations in its environment, i.e., alterations induced by the parasite. Whether the parasite-induced effects are beneficial or harmful depends on one's point of view and on conclusions derived from the total information available (Castro, 1976; Castro and Russell, 1985). Since the GI tract responds in a like manner to a number of diverse stimuli (Sprinz, 1962), it is not unexpected that many stimuli that initiate intesti-

nal inflammation lead to physiological responses similar to those evoked by parasites.

I am compelled to interject several questions at this point. Are the pathways leading to inflammation unique to parasitic infections? Is the induction of inflammation through the pathways described understandable only to a specially initiated group, i.e., parasitologists? Are pathophysiological changes unique to cases of parasitism? The answer to all 3 questions is a resounding "no." Given that, it should be obvious that host responses to an enteric parasite are not esoteric.

I will extend my consideration of parasite-induced changes in GI function. Specifically I will consider changes in epithelial function induced by antigenic challenge in the small intestine of sensitized hosts (Castro et al., 1987; Harari et al., 1987). The hosts are rats sensitized to *T. spiralis* through infection. The antigen is that derived from infective larvae of the parasite. The functional response examined is  $\text{Cl}^-$  secretion in isolated, sensitized jejunum. Antigen-induced  $\text{Cl}^-$  secretion is measured electrophysiologically using the Ussing chamber technique (Ussing and Zerahn, 1951).

Responses to antigenic challenge are quite characteristic. Challenge of sensitized jejunum leads to a biphasic  $\text{Cl}^-$  secretory response, referred to as "fast" and "slow"  $\text{Cl}^-$  secretion and represented by a change in short circuit current. These changes are triggered by mast cell-mediated anaphylaxis. Five-hydroxytryptamine (5-HT) and histamine were identified as mediators of fast  $\text{Cl}^-$  secretion, and prostaglandin  $\text{E}_2$  ( $\text{PGE}_2$ ) as the major mediator of slow  $\text{Cl}^-$  secretion. This was done by isolating mast cell-"derived" substances which were also known to act as  $\text{Cl}^-$  secretagogues in the intestine, and by mimicking the antigen-induced response with such substances. The role of these autacoids was further implicated by the ability of specific agonists of 5-HT and histamine, cinancerin, and diphenhydramine, respectively, to inhibit fast  $\text{Cl}^-$  secretion induced by antigen. An inhibitor of endogenous PG synthesis, indomethacin, inhibited slow  $\text{Cl}^-$  secretion. Additionally it was established that the transduction of the antigenic-signal by 5-HT and histamine involved intrinsic neural pathways. Atropine and tetrodotoxin were effective blockers of fast, antigen-induced  $\text{Cl}^-$  secretion and of secretion evoked directly by 5-HT and histamine. These findings are summarized in Figure 4.

## Integration of Host Responses

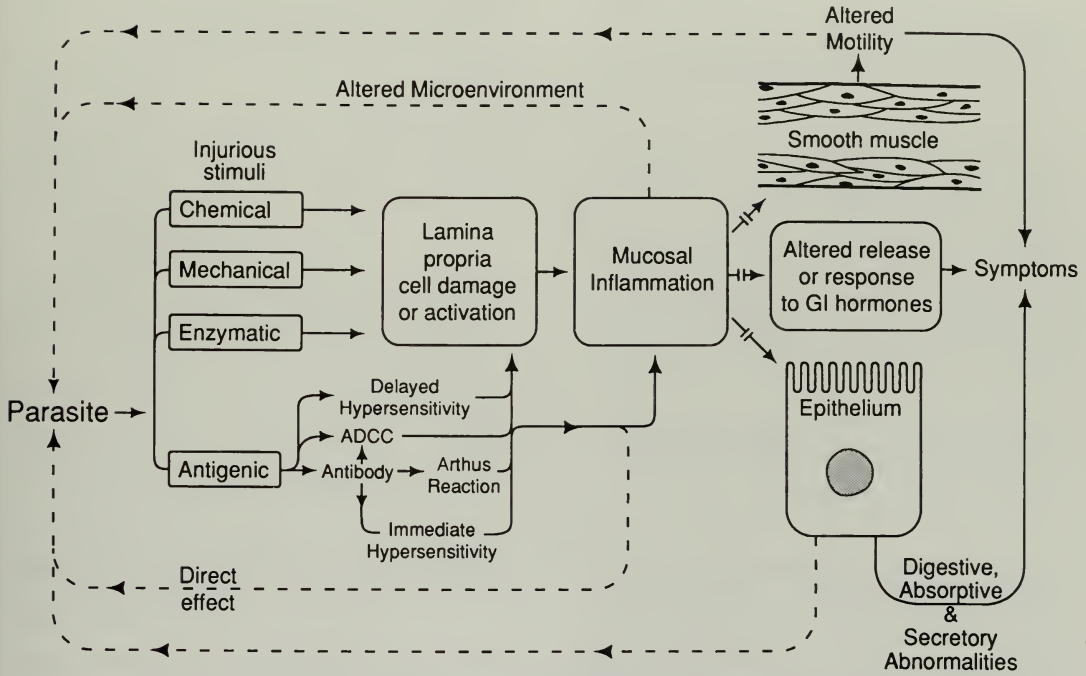


FIGURE 3. Integration of host responses to enteric stages of *Trichinella spiralis*. From Castro (1976).

Antigen-induced  $\text{Cl}^-$  secretion is triggered by a Type I hypersensitivity response. This is evident in part from the rapid onset of  $\text{Cl}^-$  secretion following antigenic challenge and in part from the passive transfer of the slow phase of antigen-induced  $\text{Cl}^-$  secretion with serum containing anti-*Trichinella* IgE. Expression of only the slow phase of  $\text{Cl}^-$  secretion in passively sensitized hosts presented an intriguing problem. Why is only the slow phase expressed? It was subsequently determined that recipients could be conditioned to express both phases of  $\text{Cl}^-$  secretion if mucosal mastocytosis was induced prior to transfer of serum containing anti-*Trichinella* IgE (Harari and Castro, 1988). In short, expression of the biphasic response requires IgE and the presence of a critical number of mast cells in the intestinal mucosa. In the passively immunized hosts mast cell numbers do not increase. The fact that the quantity of mast cells dictates the quality of the physiological response is also vividly illustrated in rats actively immunized with *T. spiralis*-derived antigen. Mast cell numbers remain low in those hosts and only the slow phase of  $\text{Cl}^-$  secretion is expressed upon antigenic challenge of jejunum.

Results from several other studies from different laboratories that are analogous to those obtained from the rat-*Trichinella* model are summarized in Table I. Common findings in the tabulated results are that (a) antigenic stimulation of sensitized mucosal tissue causes epithelial  $\text{Cl}^-$  secretion, (b) secretion is triggered by a Type I hypersensitivity response, and (c) the enteric nervous system is usually involved in the transduction of the antigenic signal (Castro, 1988a). These commonalities are evident despite differences in (a) the species of experimental animals, (b) the GI tissue being studied, (c) the homocytotropic antibody type involved, (d) the antigen used for sensitization and challenge, (e) the method of immunization, and (f) the specific mediators involved in the transduction of antigenic signals. What these studies illustrate are common regulatory pathways in a common mucosal immune system (Castro, 1988b; Heyworth and Jones, 1988), a system that responds no differently to parasite-derived antigens that it does to nonparasite antigens, i.e., these responses are not evoked only by parasite-derived antigens.

Question C: Once a host is infected with a parasite, what can be done to terminate the in-



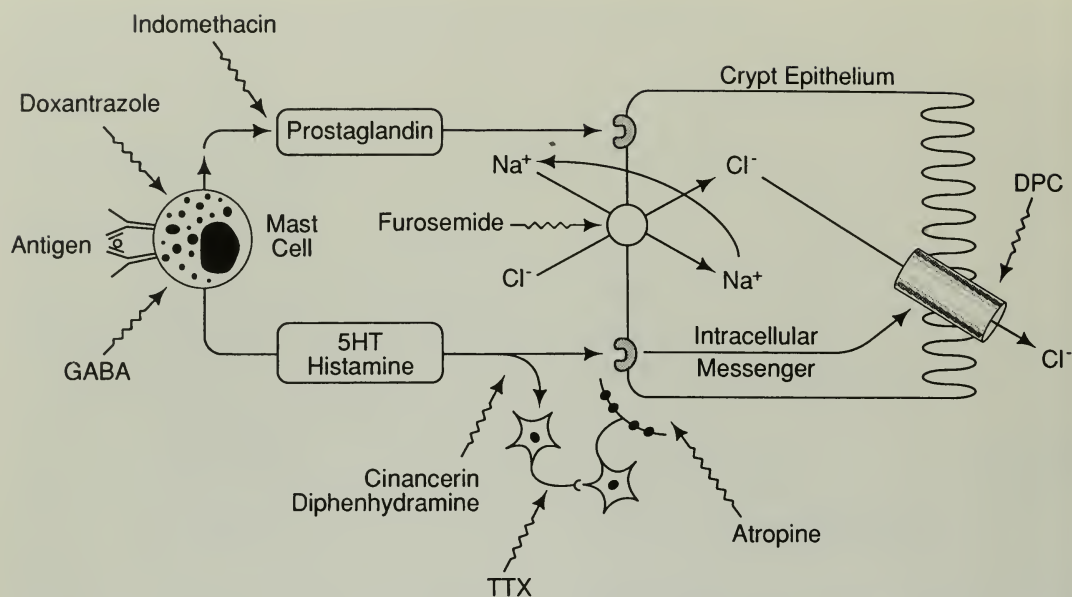


FIGURE 4. Immunophysiological basis of intestinal Cl<sup>-</sup> secretion in murine trichinosis and stepwise inhibition by pharmacological agents.

fection, alter its course, or prevent its recurrence? Answers to this question must be extracted from information on chemotherapy, vaccine development, production of parasite-resistant hosts, and environmental control of parasites. The point emphasized here relates to the previous topic. It involves the pharmacological control of physiological responses in the GI tract evoked by a parasite-derived antigen (Fig. 4). In antigen-induced Cl<sup>-</sup> secretion mediated by anaphylaxis the secretory response can be blocked by agents which (a) prevent mast cell release of mediators, (b) antagonize the action of these mediators once released, and (c) interfere with epithelial functions (see Fig. 4). I emphasize these modulatory processes in part because they are of interest to

others in related fields and not to parasitologists alone (Castro, 1988b).

Hopefully through this brief presentation you have learned something new about enteric parasites that can be passed on to others at an opportune time. At the very least, you should have learned that I do not consider enteric parasitism to be synonymous with esoteric parasitism. Also, you learned the answer to the riddle. You know who the Pariahs are. Accordingly, what does the future hold for them? As might be surmised that is a question of concern to me. In fact that question should be of concern to all parasitologists, because if the Pariahs are doing well all parasitologists should be doing well. In this regard I look to the next decade to be more exciting than

TABLE I. Anaphylaxis-evoked changes in epithelial ion transport.\*

Species (tissue)	Ig type	Antigen-evoking Δ transport (ΔI <sub>sc</sub> )	Mediator of ΔI <sub>sc</sub>	ENS involvement (ΔI <sub>sc</sub> sensitivity to atropine/TTX)	Reference
Guinea pig (colon)	G <sub>1</sub>	βLG	Histamine	None	Baird et al., 1984, 1987a
Guinea pig (colon)	—	βLG	5-HT	TTX	Baird and Cuthbert, 1987
Guinea pig (ileum)	—	βLG	—	TTX	Baird et al., 1987b
Guinea pig (colon)	—	<i>T. spiralis</i>	Histamine and others (?)	TTX	Russell and Castro, 1987
Rat (jejunum)	E	<i>T. spiralis</i>	5-HT, histamine, PGE <sub>2</sub>	Atropine/TTX	Castro et al., 1978
Rat (jejunum)	—	Ovalbumin	Histamine and others	TTX	Perdue and Gall, 1986
Rat (jejunum)	—	Ovalbumin	5-HT, PGE <sub>2</sub>	None	Catto-Smith et al., 1988

\* Abbreviations: Ig, immunoglobulin; Δ, change; ΔI<sub>sc</sub>, change in short circuit current; βLG, β-lactoglobulin; ENS, enteric nervous system; TTX, tetrodotoxin; 5-HT, 5-hydroxytryptamine; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>.

any time in the past for those who work on the fauna of the gastrointestinal tract. If for no reason other than the intellectual excitement that will emanate from systematic and integrative studies of enteric parasitism and a closer interaction with others in related disciplines, the Pariahs of the scientific caste system should be able to look back 10 years from now and realize that they took their turn at going from "rags to riches."

#### ACKNOWLEDGMENTS

Relative to the original work discussed in this address I gratefully acknowledge support from the NIAID; NIH provided in the form of critical reviews of research programs and research grant AI-11361. I thank Hassan Alizadeh, Graham Bullick, Yael Harari, Jeffrey Palmer, and Deborah Russell for their investigative efforts. I thank my faculty colleagues, past and present, in the Department of Physiology and Cell Biology, University of Texas Medical School at Houston, for their collaborative contributions, collegial support, and academic stimulation.

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## INTRODUCTION OF ALBERT DEAN BEFUS AS THE HENRY BALDWIN WARD MEDALIST FOR 1988

W. M. Kemp

Ladies and gentlemen, honored guests, members of the American Society of Parasitologists, President Castro, Vice President Murrell, and Secretary-Treasurer Mayberry, the Awards Committee is privileged to announce the Henry Baldwin Ward Medalist for 1988, Dr. Albert Dean Befus.

Dr. Befus was born on 8 October 1948 in Edmonton, Alberta. He received the B.Sc. in Zoology in 1970 from the University of Alberta, the M.Sc. in Parasitology in 1972 from the University of Toronto, and the Ph.D. in 1975 in Immunoparasitology from the Wellcome Laboratories for Experimental Parasitology, University of Glasgow. His Master's thesis was entitled "*Aspects of the Biology of *Corallobothrium parafimbriatum* sp. n. and *Corallotaenia minutia* (Cestoda: Proteocephaloidea) from the Brown Bullhead, *Ictalurus nebulosus*" and his Doctoral dissertation was entitled "Intestinal Immune Responses of Mice to the Tapeworms *Hymenolepis diminuta* and *Hymenolepis microstoma*." He served as a postdoctoral associate in the Department of Medicine at McMaster University from 1975 to 1978 in the field of Mucosal Immunology. He was an Assistant Professor (1978–1982) and Associate Professor of Pathology (1982–1985) and Co-Director of the Intestinal Diseases Research Unit of the Canadian Foundation for Ileitis and Colitis from 1984 to 1985 at McMaster. He joined the Department of Microbiology and Infectious Diseases at the University of Calgary as an Associate Professor in 1985, became a Professor and Chairman of the Immunological Sciences Research Group 1 year later, and retains those titles to the present time.*

Dr. Befus has been awarded numerous fellowships and scholarships from prestigious institutions, belongs to 10 learned societies, and has published approximately 150 papers in scholarly journals and other scientific publications. He has maintained constant and stable funding for his research program since 1979, totaling over 1.5 million dollars.

Dr. Befus' research efforts have focused upon the role of the mast cell in immune phenomena. His work has gained him worldwide recognition, particularly as it regards the physiology of these cells in relation to parasitic infections. He has made original contributions in the areas of mast cell origin, differentiation, and proliferation, while drawing attention to the value of parasite systems in approaching fundamental questions in the general field of immunology. His contri-

butions relate not only to the mast cell as an effector arm of the immune response, but as an active regulator of tissue function. Dr. Befus and his colleagues have provided evidence that mast cells display different characteristics according to the different tissues with which they are associated and that different chemical mediators may be released by different cell populations upon appropriate stimulation. This work has impacted greatly on our understanding of how immunological factors affect physiological functions in the respiratory and gastrointestinal systems. The clear demonstration of heterogeneity or pleomorphism of mast cells has brought attention to the possible analogous situation among other cell types that comprise the immune system and the potential consequences of the differences in the regulation of tissue functions, both in health and in disease states.

Dr. Befus' scientific reputation is evidenced by the receipt of over 30 invitations during the past 10 years to participate in or speak at national and international scientific or professional meetings. He has taught courses in immunology, immunogenetics, psychoneuroimmunology, and microbiology. He is known for the clarity of his teaching, as well as his enthusiasm for interaction with students.

The following is a summary quote from one of Dr. Befus' nominators and is characteristic of all letters received regarding his nomination. "It should be evident from the foregoing comments that Dean Befus is a young scientist of the highest caliber. Despite his 'youth' he has had a productive and highly meritorious career. His research work has evolved from studies of life cycles and immunity to tapeworms to mucosal immunity and mast cell biology. His finest work, which has brought him international fame, is that related to the mast cell. Although I have addressed Dean's professional strengths, I would be remiss if I did not tell you also that he is one of the most intellectually honest, honorable, and trustworthy individuals that I have known. He is truly a scholar and a kind, courteous and thoughtful person. There is no doubt that his good work and loyalty to parasitology will continue for many years and that the ASP can depend on him for leadership and as a role model to be emulated."

Based upon the above stated credentials and letters of support, the Awards Committee is pleased to introduce to you the 1988 Henry Baldwin Ward Medalist, Dr. Albert Dean Befus.



## HENRY BALDWIN WARD MEDAL ACCEPTANCE SPEECH

### A. Dean Befus

Immunological Sciences Research Group, Department of Microbiology and Infectious Diseases, Health Sciences, University of Calgary, Calgary, Alberta, Canada T2N 4N1

Dr. Kemp, President Castro, officers and members of the Society, and guests, I will remember with great joy the day that I was notified I was the 1988 recipient of the prestigious Henry Baldwin Ward Medal. It is such a thrill to receive this honor and I will cherish it with great pride, particularly because the medal is a celebration of the contributions of Dr. Ward to parasitology. He was not only instrumental in the founding of our journal and its managing editor from 1914 to 1932, but in 1924 he was the first President of the Society.

In my experience, parasitology is unique in the pride that unites its pupils, as they regularly expound upon its multi-disciplinary nature and demands on their energies and intellect. As stated by our President in the June newsletter, parasitology has a philosophy, culture, and rich heritage. This provides a basis for international cooperation and camaraderie to ensure that the challenges of continued adaptation in the face of rampant diversification, yet increasing demands for specialization, are anticipated and confronted successfully by our community and funding agencies.

I am pleased to have the opportunity to publicly express my gratitude to the various individuals who have been instrumental in my continuing education. Unfortunately, I am certain that there are many individuals whose important contributions I have been unable to recognise and I must apologize in advance for my lack of vision. Moreover, I recall the time that Dr. Ray Freeman, my M.Sc. supervisor, introduced me for a seminar at the University of Toronto after I had completed my Ph.D. studies in Glasgow. As he reviewed my training and its apparent rationale, I was struck by the different interpretations we had of past events. The point is that interpretation of the rationale of past events is constantly evolving to accommodate changing values. Therefore, at best, all I can hope to do is to give a present view of how my training proceeded.

As well as acknowledging the contributions of many individuals, I would like to describe why particular paths were selected and opportunities sought in my career development. In keeping with the unofficial theme of this meeting, "adaptation," this represents one example of an adaptation to the rigors of a career in parasitology that may provide some insight for those presently in the more formal phases of their training. Finally, I would like to make brief comments on the future of our discipline and identify some strategies that might bear fruit in the continued efforts to strive for excellence in teaching, investigation, and knowledge in parasitology and biomedical science.

My interest in parasitology arose when, at the end of my second year of Zoology at the University of Alberta where I was studying to be a fisheries biologist, I worked as a summer student with Leslie Uhazy, an M.Sc. student with Dr. John Holmes. Collecting big-



horn sheep stools all summer in many diverse and beautiful areas of the Canadian Rockies was really a joy and provided excellent topics for conversation with family and friends for a number of years. Les was a wealth of information, and with guidance from John, my path was set. I subsequently took all the undergraduate parasitology courses at U. of A. and began to seek advice about future direction. As an impressionable undergraduate student, I was strongly motivated by the enthusiasm and emphasis on quality that the parasitology students and postdoctoral fellows exemplified. Ron Podesta, Bill Samuel, and Les Uhazy were important models.

John provided the critical view, outlined the types of options available, and encouraged me to seek opportunities for growth with parasitologists in other institutions. He stressed quality and the potential of experiences in new environments, and when the time came for critical decision-making, provided gentle, but firm direction. He emphasized that we seek to adapt by incorporating into our own repertoire those qualities that we value in others but at the same time aggressively selecting against those qualities seen as weaknesses. I have certainly attempted to employ this wisdom and undoubtedly have too often expounded its virtues to students and colleagues.

In 1970 as a newly married couple, Marg and I ven-

tured off to Toronto. Dr. Ray Freeman, my supervisor, together with Drs. Desser, Fallis, McIver, and Wright in the Department of Parasitology at the University of Toronto presented a diversity of expertise and interests that complemented my earlier exposure to parasitology. Ron Podesta had moved to Toronto as well and provided support and intellectual challenge. Sherwin Desser became a great friend and always pushed for the highest levels of achievement intellectually, as well as on the running track.

My M.Sc. thesis problem involved analysis of the life history of a poorly known group of proteocephalid tapeworms of catfish, the developmental biology of their metacystode phase, and transmission. After much prodding Ray helped me realize that I had indeed been studying more than one member of the tapeworm family, one of which had not been described previously. I remember my stubbornness in accepting what subsequently became so obvious, despite the clarity of argument that Ray provided.

Ray demanded careful anticipation of research options and clarity of thought and word. I try to help my students and colleagues in their scientific writing, but can only give a fraction of what Ray provided me and so many others. It was a pleasure to listen to him so clearly present his views and their rationale on the ontogeny and relationships of cestode groups. At times we challenged his logic, but he had anticipated almost all our concerns and had a carefully considered response. I am proud to have been his student when he was putting his thoughts to paper for the publication of his critical analysis of cestode ontogeny that had evolved over more than 2 decades (*Advances in Parasitology*, 1973). Perhaps the greatest gift that Ray provided was the belief that "there is always a place for those who do well." I never believed him when he said that the opportunities will be endless and that the most serious problems would relate to the indecision surrounding which ones to grasp and grow with, without compromising your principles or losing job satisfaction. Was he right!

The challenge and excitement of new environments and people stimulated another move for Ph.D. studies. Although I originally sought a position in a graduate program in fish parasitology in the U.K., I quickly jumped at the opportunity to study immunology of worms in the mammalian intestine. This interest had grown out of an essay project on intestinal ecology for an undergraduate parasitology course and was nurtured by questions on the basis of pathogenesis in lungworm-pneumonia complex in bighorn sheep, which had arisen during my summer position in the Rockies. My Ph.D. project with Dr. C. A. Hopkins (Hoppy), University of Glasgow, was on the immunology of the lumenal-dwelling tapeworm, *Hymenolepis diminuta*, in mice. In addition to the cultural changes that Scotland presented, new scientific and academic awakenings were thrust forward that stimulated considerable, albeit insufficient, self-analysis. Our portion of the Wellcome Laboratories for Experimental Parasitology was navigated along its course by Hoppy in a close, family-like manner encompassing the technical staff, secretaries, graduate students, and new lieutenant, Dr. Derek Wakelin.

Morning and afternoon coffee and lunch were shared

by all on an equal footing. The tradition that when a new paper was published from the laboratory the first author brought in a cake for the lab to share in the celebration helped ensure that everyone experienced at least some of the rewards. Marg's Canadian chocolate cake became famous. In this family unit, no one was treated as a pair of hands doing "less important" or "less prestigious" work. All contributed critical components to the group. Hoppy led by example and Derek reinforced this signal principle.

Although it has taken some time to recognize the impact of this environment on my current approach, it is clear that in the fundamental philosophy of how I attempt to organize my laboratory this team concept, encompassing equality of worth and job satisfaction for all, is paramount. Often research technologists, secretaries, and other support staff are isolated in academic environments. I refuse to be a player in all the negative connotations that this brings forward. I guess that one has to develop some sense of self-worth combined with humility so that one does not employ strategies to inflate one's own ego which in turn destroy those of others. This is a challenge at times, especially when one encounters new environments, people, or opportunities. It is so disappointing to recognize that one has fallen into the trap yet again. How many times must I learn this lesson? Although this philosophy evolved most completely during that receptive phase in Glasgow, the same qualities were parts of the environments in both Toronto and Edmonton. Undoubtedly, the seeds, their early differentiation and partial maturation, were set by the family unit my parents established and nurtured.

As I struggled to learn immunology during my Ph.D. program and began to look to returning to North America, it was obvious that if I was to contribute to the field of immunoparasitology in the intestine, I had to do postdoctoral studies in a front-line mucosal immunology laboratory. I had developed some confidence in my training and abilities and recognized that although I had worked with exceptional supervisors, the field of study was a bit esoteric in medical sciences. It seemed obvious that I needed to move into research arenas and a research mentality that would open new avenues for career development. Accordingly, I applied for a postdoctoral position in the laboratory of Dr. John Bienenstock, McMaster University, Hamilton, Ontario, because of his international standing in the study of mucosal immunology. Fortunately I was successful and in the late summer of 1975, I commenced my first experience in the large, dynamic medical research laboratory.

Boy was I lost! I had never worked with a live cell, had no formal immunology training, and had always had close personal supervision. I was fortunate to have had strong training in basic science, otherwise I may not have gained in the same ways from this experience. After a very unproductive and threatening year trying to establish an unusual immunoassay that had been published by others, John and I decided to utilize my background in parasitology to explore the regulation and characteristics of intestine mucosal mast cells. Both of us have pursued various aspects of the biology of this cell since then. Parasitic infections of the intestine provide unique and outstanding models to help elu-



cidate the properties of this cell which is central, not only in allergic reactions, but also in many inflammatory diseases of the intestine. Parasite models have made similar important contributions to the study of IgE antibody, eosinophil function, and the modulation of inflammatory responses. It is exciting to be part of a field which is moving so quickly.

John Bienenstock is not only a fine friend but an excellent leader through the flare and confidence that he emanates. Emphasis must be on quality, effective verbal and written communication, and on networking with all the human and other resources available. Change and growth must be constants. Anything can be accomplished if you set your mind to it and opportunities are plentiful. The problem is in identifying what is a realistic target given your energy, resources, and other priorities in daily life. Sounds familiar, eh, Ray!

Leadership became an issue as I watched John and others and reviewed how my earlier mentors had approached their groups and responsibilities. I got lots of exposure to John's style as I became a faculty member in his department. We worked as a team in the study of mucosal immunology for a total of 10 years. My colleagues Judah Denburg, Jack Gaudie, and Mark McDermott became close friends, and each has stimulated me to appraise and adjust so many values that are central to my interactions with others.

I received a Rockefeller Career Development Fellowship in Tropical and Geographic Medicine in the Great Neglected Diseases Program, directed by Dr. Ken Warren. Some questioned whether the innovative vision that this program set as its goal was realistic, but the network that was established met the challenge. The annual meeting of the network was the highlight of each year for me from 1980 to 1986 as the quality of science was high, the ideas and technologies leading, and the atmosphere confident and electric. Each year the critical nature of publishing in high-impact journals in medical sciences was reinforced and excellent examples of the quality necessary were abundant. The spectrum of international scientists that became friends in a few short days each year has not been matched for me in any other forum.

The Rockefeller Fellowship allowed me to work at the Medical Research Council, U.K. Laboratories, The Gambia, West Africa, 1983. The opportunity to experience tropical diseases firsthand and to gain from the extensive experience of the Director, Dr. Brian Greenwood, and my close collaborator, Dr. Mike Rowland, was invaluable and refreshing in my career direction. New interest in research in human disease was kindled, and the value of longitudinal study design was nurtured.

In 1985 I accepted a faculty position in the Gastrointestinal Research Group, Department of Microbiology and Infectious Diseases, Faculty of Medicine, University of Calgary. There is no doubt that it is a pleasure to return to family and old friendships in western Canada. Calgary presents many new challenges and opportunities. I had forgotten how much one grows when moving into a new environment and seriously underestimated the impact of moving into a setting where one has no proven record or local credibility. I fell into the old trap of insecurity and acted through

words rather than deeds. It is advantageous that my new colleagues are understanding and patient and that Marg is such a good listener and guides me through the problems with gentle support.

The spectrum of scientists and staff that have been in the laboratory in Calgary represents a wealth of human resource, eager to learn and excited by the research problems. I dearly hope that I never lose the opportunity to interact with such refreshing and inquisitive minds: Barb, Carol, Connie, Doone, Gillian, Hidekazu, Jennifer, Lisa, Mark, Neal, Peter, Ramaswamy, Ron, Tatsuya, and Tim, and Toru. The team dictates that interpersonal skills are as important in daily activities as experimentation. The design must be that opportunities for personal growth are available and clear for all. My own personal rewards come as much from helping to provide such an environment as from the success measured by publications. This merely reflects minor modifications to a model employed by all the people who provided such an environment for me.

So what does the future hold for parasitology, and what should I strive to provide as my contribution? Most importantly, research staff, graduate students, and postdoctoral fellows must play a central part. The field is moving fast and despite concerns that one's contributions will be increasingly insignificant, I am optimistic that new students of parasitology will impose demands and provide the energies and innovation necessary to move the field forward. I will attempt to funnel my energies towards facilitating their opportunities.

Secondly, I will continue to work for increased linkages with individuals and institutions in countries where my parasitologic and other skills may aid in development programs. The critical philosophy is that such international cooperation must be based on mutual respect and opportunity and not on the outdated model of "we-the-giver." The Division of International Development at the University of Calgary is orchestrating some of these activities on campus, and given its multi-faculty approach, in recent months I have had exciting new interactions with adult educators and other social scientists who have skills that might provide innovative strategies to deal with some of the major parasitologic problems confronting us. Ray's words continue to ring true—opportunities are abundant.

It is also clear that scientists cannot isolate themselves from society. We must communicate our activities regularly and well. Lobbying at federal and provincial or state levels must be part of public awareness programs. Such objectives dictate that we become constructive in our interactions with government, rather than merely critical. I am pleased to be part of what I believe to be a major change in these types of awareness programs in Canada through the Science Policy Committee of the Canadian Federation of Biological Societies. Personal growth is a major reward for investments of time and energy in these types of activities, and I encourage others to participate more widely.

In closing, this award is an honor to receive and will be an endless source of pride. It is shared by Marg who has invested more than should be expected in any relationship and from whom, together with our children Dillon and Lindsay, I draw my conscience and joy for life.

## AMERICAN SOCIETY OF PARASITOLOGISTS

### Sixty-third Annual Business Meeting

3 August 1988

Wake Forest University, Winston-Salem, North Carolina

The Sixty-third Business Meeting was called to order by President Gilbert A. Castro at 3:00 P.M. in the Brendle Auditorium, Scales Fine Arts Center at Wake Forest University, Winston-Salem, North Carolina, on 3 August 1988.

President Castro welcomed the members and guests present and presented plaques extending appreciation on behalf of the Society to Gerald D. Schmidt for his service as Secretary-Treasurer (1981-1987) and to Sherwin S. Desser and David F. Mettrick for their service as Editors (1984-1988). President Castro then called for reports of officers. Secretary-Treasurer Mayberry presented a summary of her report to Council. Editor Desser presented a report of the Editors' office. Program Officer Duszynski and Archivist Pritchard reported on activities of their offices. These and other reports are presented in detail in the minutes of the Seventy-eighth Council Meeting.

President Castro called on Gerald Esch to present a report from the Local Committee. Dr. Esch announced that approximately 275 persons were in attendance.

President Castro called on Secretary-Treasurer Mayberry to report to the membership the significant actions of the Seventy-eighth Council Meeting, which was held 31 July 1988. Significant actions of Council were as follows:

1. The Council approved a motion to accept the ad hoc Bueding and von Brand Award Committee guidelines for selection of a Memorial Fund Award recipient beginning in 1989. This award will be given to a person who has made significant contributions to the biochemistry and/or pharmacology of parasitic helminths. Candidates will be nominated by the membership and selected by the Bueding and von Brand Memorial Endowment Fund Committee. An endowment fund will be established using \$11,000 currently available from pharmaceutical firms and additional funds will be solicited.
2. A motion was passed to modify the guidelines for selection of the Henry Baldwin Ward Medalist. The modifications include elimination of the birth date requirement and inclusion of the statement that normally the award is given to a person who has not reached their 40th birthday.
3. The Council passed a motion allowing the Secretary-Treasurer to pursue the engagement of a professional financial advisor in order to invest the Society's money in guaranteed but higher yield funds.
4. The Council approved permanent expenditures for the Stoll-Stunkard Endowment Fund Lecturer of \$2,000, \$500 for publication of the lecture, and \$2,000 for the President's Symposium.
5. A motion was approved to instruct the ad hoc

Career Opportunities Booklet Committee to proceed with the printing of 4,000 booklets at an estimated cost of \$5,860 which would be from Category II funds.

6. Council approved the Meeting Site Committee's recommendations for 1992, 1993, and 1994 sites. The 1992 site—University of Pennsylvania; 1993—Atlanta, Georgia, joint meeting with the American Society of Tropical Medicine and Hygiene; 1994—Colorado State University.
7. Council empowered the current President to appoint an ad hoc committee to formulate guidelines and procedures for travel grants for students participating in the national meeting. These will be in place for the 1989 call for papers.
8. Council voted to present certificates to students who give meritorious paper presentations but are not selected for the outstanding student paper presentation award. The maximum number of certificates of merit awarded in 1 year may not exceed 10% of the total number of student papers presented in that year. A motion was also approved to present certificates to the outstanding student paper presentation awardees along with \$250 they normally receive.
9. Council voted to approve the request that the Parasitology Section of the Canadian Society of Zoologists be an affiliate of ASP.
10. Brent Nickol was recognized as the new Editor of the *Journal*.
11. Nineteen applications for membership (#5466-5484), duly nominated and seconded, were approved by Council, bringing the total of new memberships to 63 for the year.
12. Council voted to change the ad hoc Special Student Awards Committee status to that of a standing committee entitled the Student Awards Committee.

President Castro called for reports of the following committees:

1. *Tellers* (Patricia C. Augustine, Chairman; Harry D. Danforth; and Michael D. Ruff)

Patricia Augustine reported the results of the 1988 election as presented in the minutes of the Seventy-eighth Council Meeting.

2. *Auditing Committee* (George L. Stewart and J. Richard Seed)

The Auditing Committee report was read by Secretary-Treasurer Mayberry. "We have examined the petty cash accounts of the American Society of Parasitologists, and have found them to be in order."

The report was ordered placed on file.



3. *In Memoriam Committee* (Charles E. Tanner, Chairman; Rudolph Boisvenue; and George L. Stewart)

Secretary-Treasurer Mayberry reported the following deaths:

DR. AUGUSTUS BURNS WEATHERSBY, on 26 January 1988;

COLONEL H. E. SHORTT, on 9 November 1987

President Castro requested a moment of standing silence in memory of our departed colleagues.

The report was ordered placed on file.

4. *Resolutions Committee* (Sherwin S. Desser and Annie K. Prestwood)

WHEREAS, the black and the gold of the mighty Deacons beckoned, Parasitologists—come South, to the old North State, to the cities of Winston and old Salem, where the skies are forever Carolina blue, and where at night if you listen carefully, you can hear in the distance the lonesome wail of the wolfpack on the track of the Blue Devils or perhaps of the Mountaineers.

WHEREAS, Wake Forest University did provide us with food, and lodging, and facilities on their beautiful campus, home of magnificent trees, green grass, and splendid architecture.

WHEREAS, the Local Arrangements Committee ably guided by Gerald Esch and Ray Kuhn and assisted by other valuable members did provide us with all the amenities necessary for a successful meeting including beer, real cream for the coffee, iced tea, and wine coolers.

WHEREAS, numerous staff and students from Wake Forest and neighboring universities gave generously of their time and efforts.

WHEREAS, President Gil Castro and other officers of the Society, and in particular program officer Don Duszynski, symposium speakers, and other speakers did provide us with an outstanding scientific program, and

WHEREAS, American Cyanamid, Burroughs Wellcome, Eli Lilly, Fermenta Animal Health, and Upjohn graciously provided financial support for the meeting,

NOW THEREFORE, BE IT RESOLVED THAT the American Society of Parasitologists issue a "hearty" thank you to each and every person and corporation who contributed to planning, arranging, and supporting this very successful 63rd Annual Meeting.

The report was ordered placed on file.

Under Old Business, the following meeting sites were announced by Secretary-Treasurer Mayberry:

1989—Vancouver, Canada; University of British Columbia

1990—East Lansing, Michigan; Michigan State University

1991—Norman, Oklahoma; University of Oklahoma

- Under New Business, the following items were discussed:

1. It was moved and seconded that the ad hoc Special Student Awards Committee be made a standing committee entitled the Student Awards Committee. The motion was unanimously approved by the quorum present.

2. The results of the student paper presentation competition were announced by Janice Moore, Chairman, Special Student Awards Committee. The two awards for outstanding student papers were given to:

*John Barta*, University of Toronto, "Phylogeny of Apicomplexans of the Class Sporozoa," and

*Alice Wattam*, University of Wisconsin, "Genetic Control of Filarial Worm Development in Defined Strains of *Aedes aegypti* Mosquitoes."

A Certificate of Merit was awarded to S. Ciesielski, University of North Carolina, for his presentation "Intestinal Parasites in Adult Migrant Farmworkers."

3. Dr. Brent B. Nickol announced that John Barta is ASP's 1988 Nominee for the J. Roger Porter Award, given by the U.S. Federation of Culture Collections.

4. President Castro announced that Dr. Brent B. Nickol will assume editorship of the *Journal* as of 1 January 1989.

5. A discussion was held regarding allowing students to submit abstracts for poster papers 30 days prior to the annual meeting. Program Officer Duszynski indicated he would attempt it for the 1989 meeting but was charged by President Castro to take into consideration feelings voiced against the proposition.

President Castro thanked the Society members for their support and expressed his appreciation of being elected Society President. He also expressed appreciation for the work of the Local Committee, Officers, and Council Members-at-Large. President Castro then turned the gavel over to President-Elect W. Michael Kemp.

President-Elect Kemp declared the Sixty-third Annual Business Meeting adjourned at 4:45 P.M.

Respectfully submitted,  
**Lillian F. Mayberry**  
Secretary-Treasurer

## AMERICAN SOCIETY OF PARASITOLOGISTS

### Seventy-eighth Annual Council Meeting

31 July 1988

Wake Forest University, Winston-Salem, North Carolina

The Seventy-eighth Council Meeting convened at 9:00 A.M. in the President's Boardroom, Reynolda Hall at Wake Forest University, Winston-Salem, North Carolina. Attending were President Gilbert A. Castro, President-Elect W. Michael Kemp, Immediate Past President William C. Campbell, Vice President K. Darwin Murrell, Secretary-Treasurer Lillian F. Mayberry, Editor Sherwin S. Desser, Program Officer Donald W. Duszynski, Archivist Mary Lou Hanson Pritchard, and Council Members-at-Large Lawrence R. Ash, Burton J. Bogitsh, Daniel R. Brooks, Raymond T. Damian, Raymond E. Kuhn, and J. F. Williams. Also attending were Stephen G. Kayes, Willis A. Reid, Larry Roberts, Howard J. Saz, Jack Bristol, Brent Nickol, Janice Moore, Ralph Lichtenfels, Rick Cawthorn, Sharon Patton, John S. Mackiewicz, Gerhard Schad, and Hilda Ching.

President Castro called the meeting to order and welcomed those present.

The minutes of the Seventy-seventh Council meeting were accepted as published in Volume 74, Number 1, of *The Journal of Parasitology*.

George L. Stewart and J. Richard Seed were appointed by President Castro to act as the Auditing Committee and the President appointed Annie K. Prestwood and Sherwin S. Desser to serve as the Resolutions Committee. President Castro recognized Gerhard A. Schad, President-Elect for 1989 and new Council Members-at-Large for 1989-1992, William L. Current and Robert B. Grieve. He expressed appreciation to outgoing Council Members-at-Large, Dickson D. Despommier (1985-1988), W. Michael Kemp (1985-1988), Raymond T. Damian (1988), and Daniel R. Brooks (1988). Expression of appreciation was also given to the outgoing Editors, David F. Mettrick and Sherwin S. Desser, as well as the outgoing Nominating Committee.

#### I. REPORTS OF OFFICERS

##### A. President (Gilbert A. Castro)

The opportunity to serve as President of the American Society of Parasitologists will always be reflected upon with a feeling of gratitude, appreciation, and loyalty. The role that I played in that capacity, I hope, will be judged in most part by the quality of the Sixty-third Annual Meeting. The credit in that regard unambiguously belongs to the Program Chairman, Donald Duszynski, the Secretary-Treasurer, Lillian Mayberry, and the Local Arrangements Committee chaired by Gerald Esch and Raymond Kuhn. Gratitude is extended to council members for their wisdom and efficiency in dealing with business of the Society and to chairpersons and members of all standing and ad hoc committees for their concern and efforts on behalf of the ASP during the past year.

My report alludes to the strengths of our Society and

dwells on 3 suggestions for improving its current status. The strengths are those described in my recent message to membership published in the June 1988 ASP Newsletter. The essence of the message was that we have weathered a period during which significant concerns prevailed regarding the future of parasitology and, therewith, our Society. I am quite sure that the vitality of our field and of the ASP is no longer a major concern in the minds of most members. However, we should not be satisfied with our present state of affairs, no matter how solid.

To maintain our current momentum we must clearly and consciously focus on our purpose—to advance knowledge in parasitology through teaching and research. Societal endeavors that contribute in a major way to that purpose are, unambiguously, our journal activities and our meetings.

*Meetings:* Judging from the program, the 63rd Annual Meeting should be a resounding success both scientifically and pedagogically. It should serve as a standard to which future meetings are compared. Thus, we have a good model to follow and to try and improve upon in this arena.

*Journal:* Our journal is 1 of 3 items of concern that I raise before Council for future consideration, discussion, and action. This particular concern is not new. As I wrote Ray Damian in his capacity as Chairman of the Editor Search Committee, I feel that the *Journal of Parasitology* is our Society's most important resource in fulfilling our purpose and that the *Journal* Editor holds the most important office in the ASP. The *Journal* reflects the quality and image of our society, not only to our membership, but to the world. In that regard I submit that its quality can be improved and, therewith, the image that we project as a Society. Our goal should be to strive for world leadership in our field. The only way to do that is to strive to publish the journal that best serves and reflects the excitement, importance, and best scientific endeavors in our field. To initiate movement toward that goal I submit the following 3 recommendations.

1. Follow the course of action that emanated from discussion at the 62nd Annual Council Meeting relative to sectionalizing the *Journal* and appointing associate editors for each section. In that regard attempts should be made to appoint renowned scientific leaders who will, themselves, publish in the *Journal* and aggressively solicit papers for publication from the world's experts.
2. Remove the burden of financial and business responsibilities related to the *Journal* activities from the Editor so that he/she can give undivided attention to the scientific and aesthetic aspects of our official organ. This could be accomplished by broadening the responsibility of the Business Ad-



visory Committee to include publication and related business and financial matters. In short, rename the committee the Business and Publications Committee and charge it accordingly. To facilitate the functioning of the committee, the past and current Secretary-Treasurer and past and current Editor might be appointed as members of that group.

3. Adhere to strict publication deadlines.

*Priorities for ASP:* Although Council has dealt wisely with this issue in the recent past, it is one that must be of continuous concern. In order to aid the committee charged with addressing this subject I suggest that as a working objective that the committee focus on issues that impact directly on our purpose and attempt, each year, to present to Council at least 1 priority issue with a specific plan of action to be pursued. To assure propitious action in this area, we need to deal with important issues in a serious and timely manner, but in reasonable proportions.

*Joint ventures with other societies:* One of the primary aims of our teaching efforts in parasitology should be to teach the philosophy of our field to outsiders—nonmembers. One way to do this is to interact with other societies. This does not necessarily require holding joint meetings. Since many ASP members are also members of other national scientific organizations, it would seem appropriate to invite members of those organizations to participate in symposia that are organized by ASP members. I suggest that we attempt to entice other societies to cosponsor, scientifically and financially, portions of our meeting. This could add immensely to the prestige of the ASP while contributing to our teaching mission.

I suggest that such ventures be funneled through the Society President who would make initial contact with the President of the sister society involved. Furthermore, I convey from experience that contact should be made during the early planning stages of such ventures so that all parties concerned are given the opportunity to contribute to the organizational aspects of the joint venture.

The report was ordered placed on file.

## B. Secretary-Treasurer (Lillian F. Mayberry)

### *Communications*

Communications from the Secretary-Treasurer's office to the Membership from July 1987 through June 1988 included:

1. A call for nominations for the Henry Baldwin Ward Medal.
2. A report from the Nominating Committee including biographical information on the candidates for 1988 Officers, Council Members-at-Large, and Nominating Committee, including a mail-in ballot.

Communications with the Officers and Council Members-at-Large that required action included:

1. Two lists, totaling 22 applications for membership in the Society from July 1987 to 31 December 1987, and 5 lists, totaling 41 applications for membership in the Society from 1 January 1988

to 1 July 1988, making a combined total of 63 new members. All were approved.

2. Approval to appoint the following persons to the Meeting Site Selection Committee (1992)—George Hillyer (Chairman), Sherwin Desser, and William Marquardt.
3. Approval to establish a new ad hoc Committee, entitled the Bueding and von Brand Memorial Fund Award Committee, consisting of Howard Saz (Chairman), James Bennett, Austin MacInnis, William C. Campbell, John Richard Seed, and Ben G. Harris.
4. Approval for a plaque of appreciation to be awarded to G. D. Schmidt for his 7 years of service to the Society as Secretary-Treasurer.
5. Approval of the President's Symposium expenses for 1987 and 1988.
6. Approval to establish a new ad hoc committee, to revise and combine the "Careers in Parasitology" and the "Opportunities for Graduate Training in Parasitology" booklets. The Committee is comprised of John R. Bristol (Chairman), Albert G. Canaris, and Lillian F. Mayberry.
7. Approval of the Henry Baldwin Ward Medalist, Dr. A. Dean Befus, University of Calgary.
8. Approval of the 1988 Stoll-Stunkard Endowment Fund Lecturer, Dr. Peter W. Price, Northern Arizona University.
9. Approval to have the Society lend support to the request that the Postmaster General of the United States issue a stamp commemorating the historical work of Theobald Smith and Frank Kilbourne.

### *Membership*

As of 1 July 1988 the Society had a total membership in good standing of 1,377. This includes 1,063 Active Members, 22 Active Nonsubscribing Members, 168 Student Members, 53 Retired Members, 45 Retired Nonsubscribing Members, 14 Emeritus Members, and 12 Honorary Members.

This total compares with a total membership in July 1987 of 1,418, down 41 members (3%). In May 1986 the total membership was 1,457, down 80 members (6%), and in 1985 the total membership was 1,375.

In addition to general memberships, as of 1 July 1988 there were 1,524 Institutional Subscribers to *The Journal of Parasitology*, up 15 subscriptions (1%) from the previous year.

As of 1 July 1988 there were 145 Members not paid and 182 Subscribers not paid (numbers not included in above figures).

Council approved 53 applications for membership in 1980, 116 in 1981, 96 in 1982, 128 in 1983, 93 in 1984, 116 in 1985, 113 in 1986, 90 in 1987, and 44 through July of 1988.

### *Financial records*

1. The financial records of the American Society of Parasitologists were prepared by Weatherwax & Roark, P.A., Lawrence, Kansas. The 1987 audit was conducted by Mize, Houser & Company, P.A., Lawrence, Kansas.
2. A statement of financial condition, summarizing current assets, liabilities, and fund balances at 31

TABLE I. *American Society of Parasitologists, Inc. statement of financial condition, 31 December 1985, 1986, and 1987, and 31 May 1988.*

	1985	1986	1987	31 May 1988
<b>Current assets</b>				
Checking	\$ 58,067	\$113,448	\$ 49,349	\$483,426
Cash investments	293,121	313,611	346,560	
Petty cash	467	65	151	
Receivable—returned equip.			1,733	
Restricted				
Cash investments	48,678	53,258	48,461	
Due from other funds	5		354	90
Equipment			1,884	3,768
	<u>\$400,338</u>	<u>\$480,382</u>	<u>\$448,492</u>	<u>\$487,283</u>
<b>Liabilities</b>				
Category I	\$5	\$0	\$ 0	\$ 0
Category II	0	0	354	463
Category III	—	0	0	0
	<u>\$5</u>	<u>\$0</u>	<u>\$354</u>	<u>\$463</u>
<b>Fund balances</b>				
Category I	\$122,414	\$124,619	\$123,120	\$123,120
Category II	229,241	302,505	271,231	271,230
Category III	48,678	53,258	53,787	53,787
Receipts over expenses				38,683
	<u>\$400,333</u>	<u>\$480,382</u>	<u>\$448,138</u>	<u>\$486,820</u>
Net worth	<u>\$400,338</u>	<u>\$480,382*</u>	<u>\$448,492</u>	<u>\$487,283</u>

\* After adjustment of \$106,718 in Annual Meeting Fund Balance reported in 1986 audit.

December 1985, 1986, and 1987, and 31 May 1988 is given in Table I. The Society's net worth at the end of May 1988 was \$487,283, compared with \$488,646 in May 1987.

3. A written statement was prepared by Weatherwax & Roark in response to questions from the Business Advisory Committee regarding certain wording on the monthly balance sheets.

#### Miscellaneous

1. The transition from G. D. Schmidt to L. F. Mayberry began in September 1987 and was completed in March 1988. L. F. Mayberry made a trip to Allen Press in September for orientation regarding aspects of the business office.

2. An IBM Personal System 2 Computer was purchased by the Society for use by the Secretary-Treasurer's office. All of the membership data information is now filed in the computer as well as at Allen Press.

3. Allen Press has submitted a renewal program designed to increase our renewal rates. Beginning in 1989, Visa and Mastercard may be used as a means of payment for dues. This was done to facilitate payments from foreign members. A \$2.50 fee will be assessed to members using this form of payment.

4. All of the new bank accounts for the Society have the signatures of Society officers in addition to that of the Secretary-Treasurer. This was done to protect the Society in case of the untimely death of the Secretary-Treasurer.

5. An insurance bond of \$200,000 has been acquired by the Society through The Hartford Insurance Group. This blanket bond covers the President, President-Elect, and Secretary-Treasurer of the Society.

6. As of 19 December 1987, the Society had 60 outstanding invoices for reprints and page charges. These invoices, dating back to 1979, totaled \$6,183.77. In a final attempt to clear up the past-due debts, letters were mailed on 17 February to each individual. The response was as follows: 5 letters returned, unable to locate; 3 letters received, waiting for responses/checks; 5 invoices voided for various reasons, totaling \$483.98; and 7 invoices paid, totaling \$826.81.

There has been no response to the remainder of the 40 letters mailed.

7. The mailing lists of the Society have been made available to various publishing companies for a \$150.00 one-time use charge. Income from the rental of the mailing lists totals \$810.00 from 1 January 1988 to the present.

The report was ordered placed on file.

#### C. Editors (David F. Mettrick and Sherwin S. Desser)

As our 'editorial' days dwindle down, we'd like to summarize some of the highlights of our experience. Happily, our swan song is an upbeat tune. Most of our primary objectives have been fulfilled. These include the achievement of a greater international status for the *Journal* from the point of view of the ever-growing number of papers submitted from many different countries, the composition of our Editorial Board, our reviewers, and new overseas members.

We have attempted to maintain high standards despite the fact that this commitment to quality resulted in the rejection of more than 40% of the manuscripts received—not to mention many disgruntled authors. Certain of our initiatives, such as the minireviews, crit-



ical comments, and letters to the editors, have met with favorable response. The revised format for Research Notes, initiated this year, is being well received. We leave the editorial office with the *Journal* up to date and with virtually no backlog of manuscripts.

We are grateful to Arly Allen and his colleagues at Allen Press for their competence, encouragement, and goodwill during the past 5 years. Their unflagging support made our job less hectic than it might have been.

We also thank the Executive Officers and other members of the Society for their support and occasional kind words. The wise council of our consultants and Editorial Board members is gratefully acknowledged. Special thanks to our countless thoughtful reviewers and to Megan Paul and Henry Hong who helped with the "office work."

Finally, we wish our successor, Brent Nickol, good health, great strength and wisdom, much patience, and more tolerance and humor than he ever believed possible. We shall do everything in our power to make the transitional period as smooth as possible for Brent and the Society.

The report was ordered placed on file.

#### D. Program Officer (Donald W. Duszynski)

The 63rd Annual Meeting of the American Society of Parasitologists will be held 31 July through 4 August 1988 on the Wake Forest campus in Winston-Salem, North Carolina. Three events will occur on Sunday, 31 July. ASP Council will hold its annual meeting on Sunday morning and the Coccidiosis Conference will convene Sunday afternoon. On Sunday evening, we will initiate a new event aimed to encourage ASP Graduate Students to play a more meaningful role at our Annual Meeting. The intent of the Graduate Student Workshop is to provide our students with a forum during our Annual Meeting at which they can discuss any topic they may consider relevant or interesting. The topic of the first Workshop is "The Grants Game," and it was organized mainly by Dick Seed with the help of Ray Kuhn and Mike Kemp. Upon conclusion of the Workshop, an informal social will allow graduate students, workshop participants, and interested faculty members to continue their discussion of ideas in a relaxed atmosphere.

The Meeting will "officially" begin at 9:00 A.M. Monday morning, 1 August, when Wake Forest University Provost Edwin G. Wilson will welcome us to the campus. Afterward, Gilbert A. Castro, President of ASP will preside at the Keynote Address to be given by Kenneth S. Warren, Director, Health Sciences Division, The Rockefeller Foundation. Dr. Warren's address is entitled "The Evolution of Parasitology."

In addition to the Keynote Address, the Meeting of the Society will consist of 156 presentations. These include 3 each at the Coccidiosis Conference and the Graduate Student Workshop, 4 at the President's Symposium on "The Gut Environment in the Parasitized Host," the Stoll-Stunkard Lecture "Parasites in the Evolutionary Biology of Their Hosts," by Peter W. Price, the Presidential Address by G. A. Castro entitled "Pariahs of the Scientific Caste System," 11 in the ecology symposium "Patterns and Processes in Parasite Communities," and 5 in the immunology symposium "Comparative Immunology of Parasitic Infec-

tions." Also included are 31 posters and 97 oral presentations in 11 paper sessions covering the topics of ecology, immunology, physiology and biochemistry, invertebrate life cycles, pathology, and epidemiology. The program will also include presentation of the Henry Baldwin Ward Medal to Dr. A. Dean Befus, University of Calgary, Alberta, the Annual Business Meeting, and the annual banquet. Unfortunately, the 5th Annual R. Barclay McGhee Memorial Lecture will not be presented this year. The speaker was to be Professor Augustus B. Weathersby, Professor Emeritus, University of Georgia. Dr. Weathersby died on 26 January 1988.

This is the third year that the Annual Program will include a Student Paper Competition; 2 sessions are devoted to this competition which will include 20 presentations. The 2 winners of the Student Paper Competition will be announced after the presentation of the Henry Baldwin Ward Medal on Wednesday afternoon.

The Local Committee has arranged for an evening reception and barbecue at the Stroh's Brewery, and the price of a ticket will include transportation and food with beverages donated by Stroh's.

Camera-ready copy of the *Program and Abstracts* was mailed to Allen Press on 3 May and proofs were received on 13 May. Corrected proofs were returned to Allen Press on 17 May, and it is expected that final copy of the *Program and Abstracts* will be mailed to the membership on 9 June (3rd class, bulk mail). Copies of appropriate abstracts were mailed to those chairing sessions on 20 May along with a list of helpful suggestions on the do's and don't's of how to make each session meaningful. About 15% of all authors submitted self-addressed stamped postcards on which they could be informed of the time and date of their presentation. These were mailed after final corrected copy went to Allen Press on 17 May.

The report was ordered placed on file.

#### E. Archivist (Mary Hanson Pritchard)

Committee reports and meeting minutes were reviewed to obtain the name of the company that struck the 2 versions of the Henry Baldwin Ward Medal (The Medallion Art Company, Danbury, Connecticut), and the information was relayed to the Secretary-Treasurer. Additional records and some important correspondence have been received and added to the Society's Archives.

The report was ordered placed on file.

## II. REPORTS OF STANDING COMMITTEES

#### A. Awards (Catherine A. Crandall, Chairman; Lawrence R. Ash; Ronald Fayer; W. Michael Kemp; and J. F. Williams)

The committee received 4 nominations for the Henry Baldwin Ward Medalist. Two nominees were not considered by the Committee. One nominee exceeded the age limit by 4 days and the other was an inactive member of the Society (not paid) for 1984, 1985, and 1986.

The committee recommended the nomination of Dr. A. Dean Befus as the recipient for the Henry Baldwin Ward Medal for 1988.

The report was ordered placed on file.

**B. Business Advisory** (John S. Mackiewicz, Chairman; Danny D. Cox; Paul H. Silverman; Gerald D. Schmidt; and Lillian F. Mayberry, ex officio)

The Society continued to increase its net worth in 1987 from \$480,382 at the end of 1986 to \$448,492, or a gain of 17%, for 1987. A 21% decline in revenues from membership dues and a 77% increase in publication costs largely contributed to the less than 20% gain in 1986.

Members of the Committee have been reviewing the long (17 pages) monthly financial statements provided by the accounting firm and have not found any apparent discrepancies. The accounting firm has called attention to the fact that the financial statements "... omit substantially all of the disclosures required by generally accepted accounting principles." The Committee has recommended to the Secretary-Treasurer that the accounting firm be asked to identify such disclosures so that future financial statements can be made more complete.

In March (1988), the Secretary-Treasurer requested the Committee's advice regarding a proposal to reinvest approximately \$125,000 of Category II funds of maturing CD's from banks in Greeley, Colorado. Numerous questions have been raised about the proposal since it deviates from past Society practices. Based on consultation of the chairman with 2 university officials involved in financial and investment matters, this committee has recommended that before any action is taken on the proposal further details be clarified and that if it is implemented, all agreements and conditions be written and reviewed by legal counsel to ensure that the interests of the Society are upheld. As of this report (1 May 1988) the proposal was still under consideration.

The report was ordered placed on file.

**C. Education** (Larry S. Roberts, Chairman; Dickson D. Despommier, John C. Holmes, Hugo A. James, Gayle P. Noblet, and Gerhard A. Schad)

The Education Committee has nothing to report.

**D. Honorary and Emeritus Members** (John Janovy, Jr., Chairman; M. Dale Little; and Austin J. MacInnis)

The Honorary and Emeritus Members Committee has no nominations for Honorary or Emeritus membership or for commemorative issues of the *Journal*.

The report was ordered placed on file.

**E. In Memoriam** (Charles E. Tanner, Chairman; Rudolph Boisvenue; and George L. Stewart)

Dr. Augustus Burns Weathersby, Emeritus Professor of Entomology, University of Georgia, Athens, died on the 26th day of January 1988. Dr. Weathersby took his degree from Louisiana State University and joined the Department of Entomology in Athens after he retired from the U.S. Navy. He had a sincere, bright curiosity concerning the mechanisms by which some malaria parasites, notably *Plasmodium galinaceum*, are prevented from developing in some species of mosquitoes. The span of his interest was, however, very broad since it also covered music, painting, and athletics. Professor Weathersby, a member of the Society since 1959, was to have been the 5th R. Barclay McGhee Lecturer at the 1988 Annual Meeting of the American

Society of Parasitologists. He is survived by his wife and 2 sons.

Colonel H. E. Shortt, C.I.E., M.D., D.Sc., D.T.M.&H., F.R.S., an honorary member of the American Society of Parasitologists since 1948, died peacefully in his sleep on the 9th of November 1987, in his 100th year. Dr. Shortt, retired from the Indian Medical Service, was Professor of Protozoology and former Director of the Department of Parasitology of the London School of Hygiene and Tropical Medicine and President, in its 49th year, of the Royal Society of Tropical Medicine and Hygiene. Professor Shortt contributed enormously to public health by his classical studies of the epidemiology, transmission, and control of kala-azar; he was also instrumental in proving that *Phlebotomus* is the vector of visceral leishmaniasis and, with his colleagues P. C. C. Garnham, R. S. Bray, and N. H. Fairley, established the life cycles of *Plasmodium cynomolgi*, *P. vivax*, and *P. falciparum*. Parasitology and public health owe a very large debt to Professor Shortt and to his dedication and insight. Professor Shortt is survived by his wife, son, and daughter.

The report was ordered placed on file.

**F. Meeting Site Selection** (George V. Hillyer, Chairman; Sherwin S. Desser; and William C. Marquardt)

Although a formal report was not submitted, correspondence was received from G. V. Hillyer by the Secretary-Treasurer's office indicating that a joint meeting with the American Society of Tropical Medicine and Hygiene in 1992 is not feasible. Dr. Hillyer is pursuing the possibility of a joint meeting in 1993.

The report was ordered placed on file.

**G. Membership** (William C. Campbell, Chairman; George A. Conder; Ana Flisser; and John W. McCall)

No new membership issues were raised. The facilitation of foreign membership continues to be a matter of concern. Consideration should be given to the acceptance of foreign currency or other means of coping with the problem of various and varying exchange rates. Possibly some form of shared membership could be arranged for coworkers in laboratories in which the cost of membership is judged to represent a real hardship. Once the administrative aspects of foreign membership have been smoothed out, the Society could call attention to its services and costs through announcements in international newsletters and journals. As always, the strength of our membership depends on our journal and our meetings. Because these are the fundamental assets of the Society, and because the Committee on Priorities in Parasitology has been revamped as the Committee on Priorities of the American Society of Parasitologists, it is recommended that the Membership Committee and the Committee on Priorities be merged into a single committee.

The report was ordered placed on file.

**H. Nomenclature and Terminology** (Mary Hanson Pritchard, Chairman; Murray D. Dailey; J. Ralph Lichtenfels; Betty June Myers; Brent B. Nickol; and Clarence A. Speer)

The committee confirmed the correct spelling of the family name, Aspidogastridae, for 2 members of the



Society who were revising a book. The information was in the *International Code of Zoological Nomenclature* and 2 discussions were available in the literature, so the question was answered without involving the members of the Committee.

The report was ordered placed on file.

**I. Nominating** (Roy C. Anderson, Chairman; Philip A. D'Alesandro; Barbara L. Doughty; John W. McCall; Charles E. Tanner; and Richard B. Crandall, Alt.)

The members of the Nominating Committee were written and asked for nominations on 28 July 1987.

The various nominations were then placed on a single sheet and the members of the Nominating Committee were asked on 15 October to rate the nominations, with the most favorable being given the lowest number. There were 10 nominations for President, 11 for Vice President, 13 for Council, and 25 for Nominating Committee. The results of the process were sent on 17 November to the Nominating Committee for any additional comments.

The Chairman then proceeded to contact the most favored individuals nominated to find out if they would stand for election to office and to obtain a curriculum vitae. Two individuals were selected to stand for President, 2 for Vice President, 4 for Council, and 10 for Nominating Committee.

On 29 January 1988 the nominations with curriculum vitae were sent to Dr. Mayberry, the Secretary-Treasurer for the American Society of Parasitologists.

The report was ordered placed on file.

**J. Public Responsibilities** (Eain M. Cornford, Chairman; Paul F. Basch; Richard B. Crandall; Leo Margolis; and Kendall G. Powers)

We have had no issues brought to our attention in the past year. No requests for service or assistance of the Public Responsibilities Committee have been tendered. The membership of this Committee wishes to emphasize their willingness to serve and/or assist the President, and the Society, in any capacity appropriate to the functions of the Public Responsibilities Committee.

The members of this committee all indicated a willingness to continue to serve on this committee (at the discretion of the President and President-Elect) in the 1988-1989 year.

The report was ordered placed on file.

**K. Special Awards** (Brent B. Nickol, Chairman; Delane C. Kritsky; and J. Ralph Lichtenfels)

The paper entitled "Phylogenetic and multivariate analysis of nematodes (Heterakoidea: Aspidoderidae) from neotropical tuco-tucos (Rodentia: Ctenomyidae)," presented by S. L. Gardner at the 1987 ASP meeting, was selected to be the Society's nomination for the 1988 Best Student Paper in Systematics Award sponsored by the United States Federation of Culture Collections.

Materials were assembled and submitted to nominate S. J. Upton for the Skerman Prize given by the World Federation for Culture Collections. This award is for a young scientist, working in any branch of mi-

crobiology, who has made an outstanding contribution to systematics.

The report was ordered placed on file.

**L. Stoll-Stunkard Endowment Fund** (Stephen G. Kayes, Chairman; Philip T. LoVerde; Marc H. Dresden; and Timothy P. Yoshino)

The Committee, under the chairmanship of S. G. Kayes, selected Dr. Peter W. Price, Department of Biological Sciences, University of Northern Arizona, to present the Stoll-Stunkard Endowment Fund Lecture at the 1988 Annual Meeting. The Committee is now in the process of selecting a speaker to recommend to the Council for the 1989 meeting.

The Committee, in conjunction with the Parasitology Faculty of the University of Georgia, selected Dr. A. B. Weathersby to present the R. Barclay McGhee Lecture at the 1988 Annual Meeting. Regrettably, Dr. Weathersby passed away and it was decided to forego the lectureship for this year. The Committee and the Georgia Parasitology Faculty will nominate a speaker for the 1989 meeting. The Committee would like to go on record as extending its and the Society's deepest sympathies to the Weathersby family.

The report was ordered placed on file.

**M. Tellers** (Patricia C. Augustine, Chairman; Harry D. Danforth; and Michael D. Ruff)

Following are the results of the 1988 election of officers for the American Society of Parasitologists. A total of 557 ballots were counted; candidates accruing the largest numbers of votes are marked with an asterisk.

President-Elect (1989)

G. A. Schad\*

J. K. Frenkel

Vice President (1989)

A. K. Prestwood\*

R. E. Kuhn

Council Members-at-Large (1989-1992)

W. L. Current\*

R. B. Grieve\*

P. T. LoVerde

D. L. Wassom

Nominating Committee (1989)

A. J. MacInnis\* (Chairman)

L. S. Roberts\*

G. V. Hillyer\*

J. R. Seed\*

D. J. Forrester\*

S. E. Maddison\* (Alternate)

C. E. Carter

S. G. Kayes

R. Herman

J. P. Vanderberg

The report was ordered placed on file.

**N. Translations** (B. M. Honigberg, Chairman; Hisao P. Arai; F. G. Hochberg; Glenn L. Hoffman; and Leo Margolis)

Nothing of consequence to report. If appointed again, I shall have to find out more about the avenues one should follow to obtain lists of pertinent translations.

The report was ordered placed on file.

### III. REPORTS OF AD HOC COMMITTEES

#### A. Clinical Laboratory Standards (Lynne S. Garcia, Chairman; George R. Healy; and Ruth Leventhal)

See report of National Committee on Clinical Laboratory Standards.

#### B. Editor Search (Raymond T. Damian, Chairman; J. Ralph Lichtenfels; Philip T. LoVerde; Austin J. MacInnis; and Gerald D. Schmidt)

Names of possible candidates were solicited from committee members and others. Thirty were received. All were contacted for expression of their interest in the Editor position, and 7 expressed interest. A letter was sent to each, asking for their thoughts on the *Journal*, the editorship, and supporting materials and letters. All candidates responded. The materials were then made available to the Search Committee members, and their ranking of the candidates (I also ranked them) was returned to me. Results were communicated to President Castro by me (letter of 2/12/88). Dr. Brent Nickol had the highest ranking, followed closely by Dr. Peter Pappas. Later, Dr. Pappas withdrew his candidacy. Dr. Castro's letter of 5/10/88 to Dr. Mayberry indicated his recommendation of Dr. Nickol for the editorship, based on the Search Committee's ranking and other factors as mentioned. Council voted on Dr. Nickol as Editor by mail as solicited by Dr. Mayberry in memo dated 5/16/88, with outcome unknown to me at this time.

The report was ordered placed on file.

#### C. ASP/ASTMH Joint Meeting Committee

No report was received.

#### D. Local Committee 1987 Meeting (John Janovy, Chairman; Brent B. Nickol; and Mary Hanson Pritchard)

The 1987 meetings were held in Lincoln, Nebraska, on the University of Nebraska campus, jointly with the Wildlife Disease Association, 2-5 August 1987.

Three hundred thirty-nine (339) people were registered. The financial summary follows:

<i>Income</i>	
Registration and housing payments	\$23,860.96
Banquet/luncheon/WDA social tickets	1,751.50
Tours	37.50
Miscellaneous (program sales)	36.00
Total	\$25,685.96
<i>Expenses</i>	
Lodging charges	\$11,816.00
Catering (Nebraska Union: all coffee breaks, Museum reception, banquet, poster session, WDA luncheon; Chelsea's: WDA picnic)	6,076.28
Pres./Sec't.-Treas. suite (Cornhusker)	950.00
Facility rentals (Sheldon; Chesterfield's)	250.00
Auto pool	95.20
Telephone	14.04
Printing and duplicating	108.98

Conference office admin. fee (339

@ \$18)	6,102.00
Total	\$25,412.50
NET INCOME (LOSS)	\$273.46

The joint meeting with WDA presented a few problems, mainly because WDA had no local committee representative, but most of the difficulties were resolved.

The report was ordered placed on file.

#### E. Local Committee 1988 Meeting (Gerald W. Esch and Raymond E. Kuhn, Chairmen)

No report submitted.

#### F. Local Committee 1989 Meeting (Hilda L. Ching [replacing Daniel R. Brooks], Chairman)

The Local Arrangements Committee, chaired by Dr. Hilda L. Ching, works in cooperation with the University of British Columbia Conference Centre. Preparations are well enough advanced that no problems are anticipated due to the move of Dr. Daniel R. Brooks to the University of Toronto. Blocks of rooms for housing, meetings, formal presentations, a reception and poster session, and a banquet have been reserved. Preparations for registration have been discussed and no problems are anticipated. A slide show promoting the meeting will be displayed with the exhibitors during the Wake Forest meeting. The Local Committee is composed of members of the B.C. Parasitologists. During the fall meeting of that group, it is hoped that Dr. Duszynski will attend and approve the arrangements with the Local Committee and the conference centre people. One anticipated highlight will be the banquet, a salmon barbecue held on the grounds of the University of British Columbia Museum of Anthropology. The Parasitology Section of the Canadian Society of Zoologists wishes to be included in the sponsorship of the meeting.

The report was ordered placed on file.

#### G. Local Committee 1990 Meeting (James B. Jensen and J. F. Williams, Chairmen)

The Kellogg Conference Center has been reserved. Ample facilities are available, and there is a large group of parasitologists that will serve as the Local Committee.

#### H. Newsletter (L. K. [Vern] Eveland, Chairman; Bernard Fried; W. Michael Kemp; Lillian F. Mayberry; Betty June Myers; and John E. Ubelaker)

The 1987 Newsletter staff: Editor, Vern Eveland; Associate Editors, Gilbert A. Castro (President-Elect), B. Fried, B. J. Myers, L. Mayberry (Secretary-Treasurer), and J. E. Ubelaker. Affiliated Society Correspondents are SCSP—E. G. Platzer, RMCP—R. A. Heckman, SWAP—L. F. Mayberry, SSP—none, NCP—M. Norberg, NJSP—M. Doscher, AMCOP—D. M. Miller, and HELMOC—M. D. Ruff. When this report was submitted, Volume 10(1) was published and 10(2) was in preparation. No. 1 contained an article on the new Arthropod-Borne and Infectious Diseases Laboratory at Colorado State University, announcements about members and friends, the CIBA-GEIGY prize for research in animal health, Stoll-Stunkard and Membership Directory Fund contributors, a calendar



of upcoming events from WFP News, an obituary notice on Col. H. E. Shortt, and other miscellaneous announcements. Goals of the Newsletter are to publish items and editorials which: 1) are in the interest of members; 2) keep members informed about affiliates and other societies of parasitology; and 3) promote parasitology teaching, research, and clinical service.

The report was ordered placed on file.

**I. Priorities of the American Society of Parasitologists** (William C. Campbell, Chairman; Gerald W. Esch; J. K. Frenkel; J. Ralph Lichtenfels; and K. Darwin Murrell)

The well-being of the Society hinges on 2 factors—the quality of the Annual Meeting and the quality of the *Journal*. It has been suggested by members of this Committee that the location of the Annual Meeting deserves particular scrutiny. Perhaps big city hotels and big university campuses should be avoided (or used infrequently) although both have certain advantages. Meetings could instead be held on small or medium-sized campuses, where the advantages of low-cost student facilities and good lecture rooms could be retained without incurring the disadvantage of diminished interaction and *esprit* resulting from participant scatter, or the financial disadvantages of big hotels in big cities. Then again, meetings could be held in locations more exotic and more exciting than those we customarily employ. There are resort hotels and resort areas that can be taken over more or less completely by societies for meeting purposes. Members are probably familiar with highly successful meetings in such habitats, and while they may be particularly suitable for small societies, they are by no means limited to such—as demonstrated by the ASP meeting at Snowbird, Utah.

While it may go without saying that the success of our meeting depends on good local organization, good promotion (calling attention, for example, to special symposia, etc.), and a program that attracts highly committed scientists, Council may find it useful to address these matters anew and perhaps participate more directly in the early planning phase of each Annual Meeting.

Many members do not attend the Annual Meeting, but all receive the *Journal*, and the Society will prosper only if the quality of the *Journal* is high in terms of content, format, and timeliness. Council is therefore urged to continue its present commitment to exploring ways to provide the Editor's office with the greatest possible support. Consideration might be given to the possibility of sharing ideas, and even resources, with other societies.

The report was ordered placed on file.

**J. Special Student Awards** (Bernard Fried, 1987 Chairman; Cheryl M. Bartlett; Raymond T. Damian; Donald W. Duszynski; Max Grogg; Robert Herman; Allen D. Johnson; and Annie K. Prestwood)

The winners of the 1987 Special Student Awards were: Mr. Glenn E. Keitzmann, Jr., Iowa State University, Ames, Iowa, for his paper "Early events of caseous nodule formation in Ring Doves (*Streptopelia risoria*) following infection with *Trichomonas gallinae*," and Mr. Michael R. Lappin, University of Georgia, Athens, Georgia, for his paper "Diagnosis of recent

*Toxoplasma gondii* infection in cats by enzyme-linked immunosorbent assay for immunoglobulin M."

The report was ordered placed on file.

**K. Bueding and von Brand Award** (Howard J. Saz, Chairman; Ben G. Harris; James Bennett; W. C. Campbell; Austin MacInnis; and J. Richard Seed)

As approved by the ASP Council, President Castro has appointed the above ad hoc committee for the Bueding and von Brand Memorial Fund Award. The committee was charged with making recommendations to Council at the August 1988 meeting in Winston-Salem concerning the mechanism for establishing the award, and the specific guidelines to be used in selecting the awardee.

Currently, the Fund consists of \$8,500 which will be turned over to the ASP upon approval of the terms of the award. The present Fund members, who are acting independent of the ASP, are Drs. James Bennett, George Hitchings, Howard Saz, William Scheibel, and William Trager. All contributions thus far have been made by pharmaceutical firms. As of 20 June 1988, however, approximately 100 requests for donations have been sent to individuals. Therefore, the total fund presumably will increase. The present independent fund committee will cease to exist upon relinquishing the monies to the ASP.

The independent fund committee has stipulated that the award be made to an outstanding scientist for research on the biochemistry and/or pharmacology of parasitic helminths, and that no age limit be placed on the selection of the recipient of the award. Both the independent and ASP fund committee have endorsed these stipulations. In addition, both committees have been polled and endorse the following suggestions.

1. The fund will be known as the Bueding and von Brand Memorial Fund.
2. The award will be known as the Bueding and von Brand Memorial Award.
3. The Award will be presented at the annual national meeting of the American Society of Parasitologists in honor of the memory of both Dr. Ernest Bueding and Dr. Theodor von Brand.
4. The Award will be presented to an outstanding scientist for research on the biochemistry and/or pharmacology of parasitic helminths.
5. There will be no age limit in regard to the selection of the recipient. Membership in the ASP will not be a requirement.
6. The awardee will deliver a major address to the membership summarizing his/her research findings at the national meeting of the ASP. The address will be announced and scheduled in the program of the meeting.
7. Candidates for the award will be nominated by the membership at large. This will require a primary nominator and a minimum of 2 seconds. The primary nominator will be responsible for the submission of the candidate's curriculum vitae plus a summary outlining the candidate's most pertinent contributions. The seconds will be required to submit supporting letters. All nominating materials will be submitted to the Secretary-Treasurer of the ASP who will collate and forward the material to the Chairman of the

Bueding and von Brand Award Committee who, in turn, will distribute copies to the remaining committee members. Each committee member will rank the proposals in order of their selection (1 being highest). The Chairman of the Committee will receive the rankings from each member, add up the assigned numbers, and communicate the name of the awardee to the Secretary-Treasurer for public disclosure. In the event the proposed awardee cannot address the membership, the "runner-up" will receive the award. A majority vote of the committee would be necessary to determine the absence of an awardee for any given year.

8. It will be required that for the first 5 awardee selections, at least 1 member of the ASP Bueding and von Brand Memorial Award Committee be selected from the present members of the independent Fund Committee (Bennett, Hitchings, Saz, Scheibel, Trager).
9. A "Call for Nominations" for the Bueding and von Brand Memorial Award will be made by the Secretary-Treasurer shortly after each national ASP meeting. This will be necessary to allow time for committee action, notification of the awardee, and submission of the name and title of the awardee's paper for inclusion in the program and abstracts of the next meeting.
10. The Bueding and von Brand Memorial Award will comprise an honorarium, a certificate, and travel expenses for the awardee. It is proposed that money for the Award be obtained from 75 to 80 percent of the earnings of the Fund. This would allow for slow growth of the Fund which also would be increased by future donations. Since the Awardee will address the membership, the possibility should be considered that travel funds be paid by the ASP. This would allow for a larger honorarium. *In no case should the principal of the Fund be used to finance the Award.*
11. The ASP will invite additional contributions in a routine way. It has been suggested that contributions be invited from the individual membership of the ASP and whomever it deems desirable.

The report was ordered placed on file.

**L. Careers in Parasitology Revision Committee** (Jack Bristol, Chairman; Albert G. Canaris; and Lillian F. Mayberry)

The Committee has engaged a professional illustrator who has designed a cover and layout. Letters requesting photographs that would stimulate an interest in parasitology and demonstrate the diversity of the discipline were mailed to over 90 individuals. Photographs received are being catalogued by the Secretary-Treasurer's office. Text material is being written and will be reviewed and edited by John Janovy, Jr., Larry Roberts, and Gilbert Castro. A preliminary layout will be presented at the 1988 Council Meeting. A form to use in updating institutions where programs in parasitology are offered will appear in the June issue of the ASP Newsletter. This information will be returned to Larry Roberts, Education Committee Chairman, and will be compiled by the Education Com-

mittee. The final booklet produced will encompass both careers and opportunities in Parasitology.

The report was ordered placed on file.

## IV. OLD BUSINESS

### A. By-Law Amendment

A motion was made by Lillian F. Mayberry, seconded by Dan Brooks, and passed by Council that the ad hoc Special Student Awards Committee be made a standing committee entitled the Student Awards Committee. The Student Awards Committee shall comprise 5 members with terms of 2 years and shall be appointed by the President. Three members of the Committee shall be appointed in odd-numbered years, and 2 members in even-numbered years. The Chairman of the Committee shall be elected by the Committee. The membership of the Committee should reflect the various subdisciplines of parasitology insofar as possible. The Student Awards Committee shall select recipients for the Outstanding Student Presentation Awards to be given at each Annual Meeting of the Society. The Amendment will be submitted to the membership for voting at the Sixty-third Annual Business Meeting.

### B. National Committee on Clinical Laboratory Standards Membership

A discussion of NCCLS membership, which was tabled at the 1987 Council Meeting, was opened. It was the consensus of Council that the 1989 membership dues be withheld until the 1989 Council meeting at which time the Secretary-Treasurer will present information obtained from NCCLS on benefits of membership. A decision will be made at that time on continuation of the Society's membership in NCCLS.

### C. Stoll-Stunkard Endowment Fund Lecture and President's Symposium Expenses

A motion was made by Mike Kemp, seconded by Dan Brooks, and passed unanimously by Council to approve, retroactively, expenditures for the 1987 and 1988 Stoll-Stunkard Endowment Fund Lecturer (\$2,000) and publication of the Lecture (\$500). A second motion was made by Dan Brooks, seconded by Mike Kemp, and passed unanimously by Council for permanent expenditure approval for the Stoll-Stunkard Endowment Fund Lecturer (\$2,000), publication of the lecture (\$500), and the Presidential Symposium (\$2,000).

## V. NEW BUSINESS

### A. International Union of Microbiological Societies Membership

It was recommended by the Secretary-Treasurer and agreed upon by Council that the Society not join the IUMS since its efforts are directed toward microbiology rather than the diverse fields that comprise parasitology.

### B. Henry Baldwin Ward Medalist Guidelines

A motion was made by Bill Campbell, seconded by Dan Brooks, and passed unanimously by Council to modify the Henry Baldwin Ward Medalist Guidelines to include elimination of the birth date requirement and inclusion of a statement that normally the award



is given to a person who has not reached their 40th birthday. The motion also included referring the idea of instituting additional awards, such as for senior members, to the Awards Committee.

#### **C. Acknowledgment of Industrial Support for the 1988 President's Symposium**

The President acknowledged the following companies for their support and indicated that George Conder had been instrumental in helping to obtain funding:

American Cyanamid  
Burroughs Wellcome  
Eli Lilly and Company  
Fermenta Animal Health Co.  
The Upjohn Company

#### **D. Society Financial Advisor**

A motion was made by Ray Damian, seconded by Burton Bogitsh, and passed unanimously by Council to allow the Secretary-Treasurer to pursue engaging a professional financial advisor for advice in investing the Society's money in higher-yield/guaranteed funds.

#### **E. Business Advisory Committee**

The President and Secretary-Treasurer will prepare an expanded charge for the Committee that will include such items as negotiating page charges with Allen Press.

#### **F. Career Opportunities Booklet**

Jack Bristol, ad hoc Committee Chairman, presented a pasteup of the proposed booklet. A motion was made by Darwin Murrell, seconded by Mike Kemp, and passed unanimously by Council to instruct the ad hoc committee to proceed with the printing of 4,000 booklets at an estimated cost of \$5,860 from Category II funds.

#### **G. Bueding and von Brand Award**

Howard Saz, ad hoc Committee Chairman, presented the Committee's guidelines for selection of the Bueding and von Brand Award recipient and motioned that they be accepted. The motion was seconded by Dan Brooks and passed unanimously by Council. The ad hoc committee will function as the selection committee for 1989.

#### **H. Meeting Sites 1992-1994**

A presentation was made by Bill Marquardt in which he recommended to Council the following meeting sites: University of Pennsylvania (1992), Atlanta, Georgia—joint meeting with ASTMH (1993), and Colorado State University (1994). A motion was made by Mike Kemp, seconded by Larry Ash, and passed unanimously by Council to approve the sites.

#### **I. Student Awards**

A resolution was presented for the Southwestern Association of Parasitologists by Representative Jack Bristol, requesting that the Council consider establishing a grant-in-aid of research for a graduate student. After a discussion, it was the general consensus of Council that more support should be given to students and that the Society should provide travel grants for graduate students in order to encourage attendance at national meetings. A motion was made by Mike Kemp that the President appoint an ad hoc committee to formulate travel grant guidelines and application pro-

cedures for students participating in the meetings and that this be done expeditiously so that guidelines will be in place for the 1989 meeting. The motion was seconded by Dan Brooks and passed unanimously.

A motion was made by Dan Brooks, requesting that certificates of merit for student paper presentations be awarded and that certificates be presented to the 2 students receiving the cash awards for outstanding paper presentations. The motion was seconded and amended by Lillian Mayberry to read that the number of certificates of merit shall not exceed 10% of the total number of student papers in any given year. The amended notion passed unanimously.

#### **J. Program Officer Guidelines**

The Council's concensus was that the Program Officer has considerable latitude in making decisions regarding the structure of the program at annual meetings.

#### **K. Editor, *Journal of Parasitology***

The President introduced Brent Nickol, who will assume editorship of the *Journal* in January. The Council expressed strong feelings that the Society should support, in any way possible, the Editor's effort to produce the *Journal*. Council approved subsidy of Brent Nickol's expenses to visit Allen Press before January.

#### **L. New ASP Affiliate**

A motion was made by Burton Bogitsh that the request by the Parasitology Section of the Canadian Society of Zoologists to become an ASP affiliate be approved. The motion was seconded by Mike Kemp and passed unanimously.

#### **M. Applications for Membership**

A motion was made by Burton Bogitsh and seconded by Mike Kemp that 19 applicants (#5466-5484), duly nominated, be approved by Council. The motion passed unanimously. Sixty-three new members have been elected to date this year.

#### **N. Archivist**

A motion was made by Bill Campbell to approve the Archivist's request for approximately \$1,500 to hire, temporarily, a professional archivist to prepare an accessible collection. After being seconded by Larry Ash, the motion passed unanimously.

#### **O. ICOPA VII**

A motion was made by Darwin Murrell to subsidize airfare and per diem expenses for the Secretary-Treasurer to attend ICOPA in 1990 as the Society's representative. The motion was seconded by Mike Kemp and passed with 1 abstention by the Secretary-Treasurer. The President requested that Gerhard Schad explore a group rate for the membership.

### **VI. ADJOURNMENT**

President Castro declared the Seventy-eighth Council Meeting adjourned at 5:00 P.M.

Respectfully submitted,  
Lillian F. Mayberry  
Secretary-Treasurer

## IN VIVO ACTION OF THE ANTICOCIDIAL DICLAZURIL (CLINACOX®) ON THE DEVELOPMENTAL STAGES OF *EIMERIA TENELLA*: A HISTOLOGICAL STUDY

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**ABSTRACT:** Diclazuril, a new benzeneacetonitrile anticoccidial, has potent activity against various stages of *Eimeria tenella*. A single treatment of experimentally infected chickens during the prepatent phase (up to day 5) results in a complete interruption of the life cycle and oocyst shedding. The first- and second-generation schizonts show extensive degenerative changes that finally result in a complete loss of the parasitic stage. The degeneration is characterized by loss of internal structure, the appearance of many intracytoplasmic vacuoles, and incomplete merogony. The merozoites themselves show similar degenerative changes, including the presence of numerous small vacuoles in the cytoplasm. Diclazuril is also effective against both the micro- and macrogametocytes that have a ballooned appearance and lose their internal structure completely. In the macrogametocytes, wall-forming bodies either do not develop or disappear rapidly. Development of typical caecal lesions is prevented when treatment with diclazuril is initiated before large numbers of second-generation schizonts appear, i.e., day 3. It is concluded that diclazuril is lethal against both the asexual and the sexual stages of *E. tenella*. At the proposed use level of 1 ppm in the feed, the life cycle is interrupted at a very early stage and lesion development and oocyst shedding are completely prevented.

Coccidiosis control in chickens is based mainly on the prophylactic use of anticoccidials in the feed. Over the years, many potent drugs have been developed against the different *Eimeria* species (McDougald, 1982). Many of these drugs have been shown to have a specific action at more or less specific times in the life cycle of the parasites. In this regard, much information is available on how anticoccidials act against *E. tenella*. For example, the quinolones are static and act against the invading sporozoite (Ryley, 1967). Robenidine acts secondarily by killing the first generation of schizonts (Ryley and Wilson, 1971). The ionophores are primarily lethal against the invasive stages (Long and Jeffers, 1982; Mehlhorn et al., 1983), whereas other drugs, such as halofuginone and arprinocid, are also lethal but over a broader part of the life cycle (McQuiston and McDougald, 1981).

Diclazuril is a new broad-spectrum anticoccidial, belonging to the benzeneacetonitrile group, with a very high efficacy against different *Eimeria* species in chickens, turkeys, rabbits, sheep, and cattle. Laboratory trials indicated that diclazuril at 1 ppm in the feed is highly effective against all major *Eimeria* species in chickens (Vanparijs et al., 1988). Complete inhibition of oocyst excretion is obtained for *E. tenella*, *E. acervulina*, *E. maxima*, *E. brunetti*, and *E. necatrix*. Lesion development is completely prevented for *E. te-*

*nella*, *E. acervulina*, and *E. necatrix*, whereas some lesions may occur occasionally in the remaining species. This specific profile indicates that diclazuril has a rather species-specific action and that some variation has to exist with regard to its activity on the different endogenous stages.

In the present study, the site of action of diclazuril against the caecal stages of *E. tenella* was investigated in artificially infected chickens, using light microscopic techniques. The results of electron microscopic studies are published separately (Verheyen et al., 1988).

### MATERIALS AND METHODS

#### Experimental drug

Diclazuril was administered as a 0.5% premix in the feed at 1 ppm or by gavage as a single 5-mg/kg dose in a gelatin capsule.

#### Experimental animals

Day-old male Hisex chickens were purchased from a commercial hatchery and reared for 17 days in coccidiosis-free brooders. At the start of the experiment, the chickens were transferred to a coccidiosis-free floor-pen facility. They were divided into 9 groups with comparable mean body weights. The chickens were kept on fresh peat litter, had free access to drinking water, and were fed a commercial laying hen feed *ad libitum* without any anticoccidial medication.

#### Experimental design

At day 0 of the experiment, all chickens were individually infected by crop intubation with  $5 \times 10^5$  sporulated oocysts of a laboratory strain of *E. tenella* that had been maintained by serial passages in chickens for many years. Medication of the birds was done according to the time schedule as presented in Table I. In the

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various experimental groups (groups 1–7), each chicken was individually treated with a single oral dose of 5 mg/kg diclazuril by gavage at either 1, 2, 3, 4, 5, 6, or 7 days after the infection. Under the conditions of the experiment, the 5-mg/kg dose corresponded to approximately 10 times the daily drug exposure of the proposed in-feed medication at 1 ppm. The birds that received diclazuril in the feed (group 8) were medicated from day –1 until the end of the experiment on day 12. Group 9 was not treated and served as an infected, nonmedicated control group.

### Necropsy and histology

From groups 1 to 7, 2 chickens were necropsied at either 24, 48, 72, or 96 hr after treatment and at 8, 10, and 12 days after the infection. Two chickens from the control group (group 9) and 2 from those medicated through the feed (group 8) were necropsied at daily intervals. The caeca were scored clinically for lesions according to Johnson and Reid (1970) and the caecal contents were examined for the presence of oocysts beginning on day 6. Tissue samples of both caeca of each bird were collected in 10% phosphate-buffered formalin, embedded in paraplast, cut at 4  $\mu$ m, and stained with haematoxylin and eosin. They were histologically examined for the presence of developmental stages of *E. tenella*. Photographs were obtained from semithin sections (1  $\mu$ m) after staining with toluidine blue; the procedure is described in the electron microscopic study (Verheyen et al., 1988).

## RESULTS

### Developmental stages in the infected, nonmedicated control animals

The histological findings in the infected, nonmedicated control animals are described briefly and correspond well to what has been cited in the literature (Levine, 1961; Davies et al., 1963).

Intracellular trophozoites were difficult to find and were located above the cell nucleus in a small parasitophorous vacuole (Fig. 1). Numerous differentiating first-generation schizonts (Fig. 2) were found between days 2 and 4. Between days 3 and 6, very large numbers of second-generation schizonts (Fig. 4) were present. They were located in the lamina propria and in the submucosa of the caecal wall. Due to the severe inflammatory reaction, the caecal wall became thickened, resulting in haemorrhages and villus destruction. Gametocytes were found in the enterocytes of the villi and the crypts between days 5 and 12. The macrogamont (Fig. 6) had an eosinophilic cytoplasm and typical wall-forming bodies at its periphery. The microgamont was somewhat smaller and contained numerous comma-shaped basophilic microgametocytes that were located at the periphery of the cytoplasm. Oocysts were found from day 6 onward. They were eosinophilic and had a thick, clearly differentiated wall.

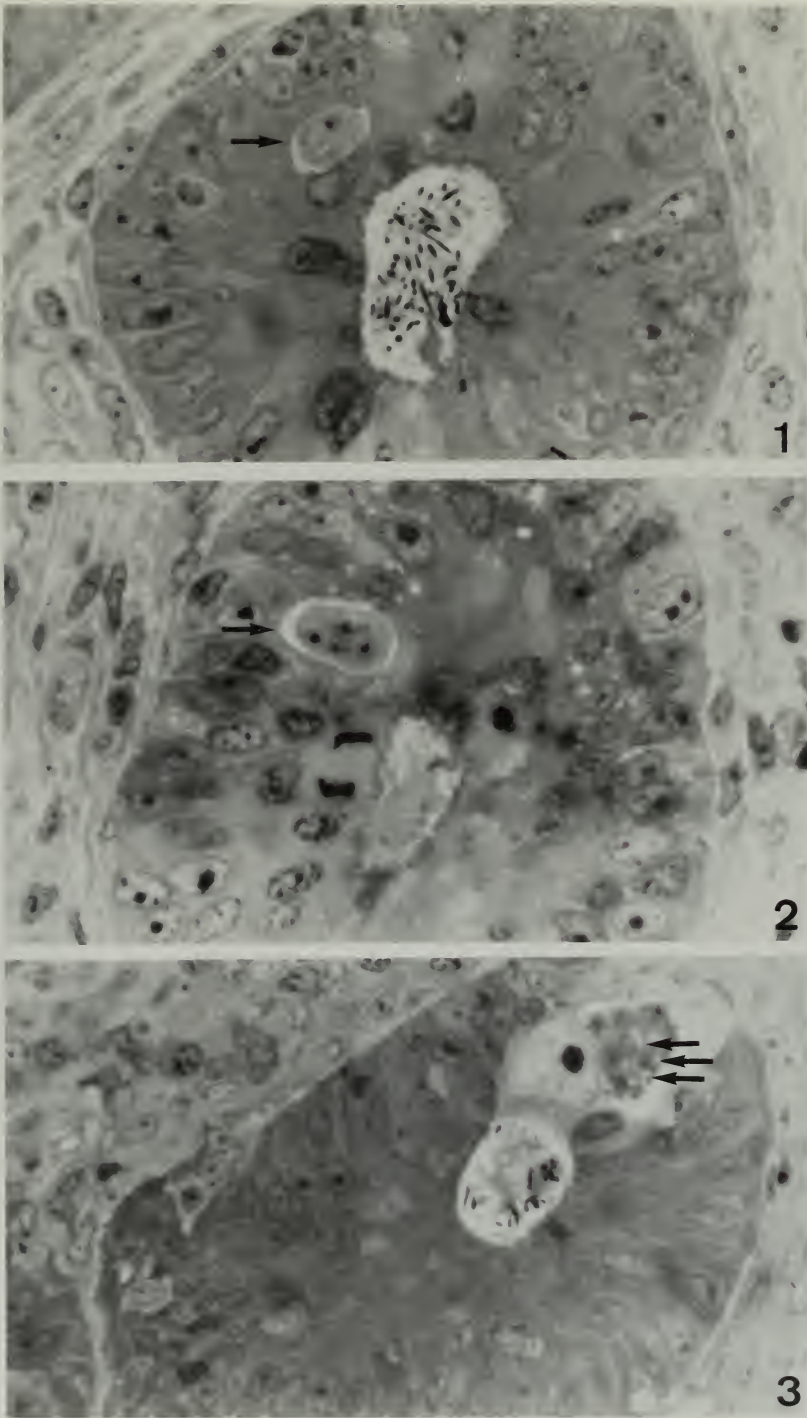
### Developmental stages in diclazuril-treated animals

In the birds treated 1 day after infection (group 1), no intracellular trophozoites were found. First-generation schizonts with merozoites were detected sporadically up to 2 days after treatment. Most were clearly degenerating, with a poorly visible wall and indistinct internal structures. First-generation schizonts were somewhat larger in size compared to undegenerated schizonts, and the parasitophorous vacuole that was always present in the infected, nonmedicated control animals had disappeared. Merozoites, if present, also degenerated and were observed as amorphous material at the edge of the schizont. The degenerating schizont gradually became less basophilic and accumulated numerous intracellular vacuoles of various sizes (Fig. 3). Obviously, the life cycle was completely interrupted, as no other endogenous stages were detected and no oocysts were formed.

Treatment on day 2 (group 2) or at the stage of first schizogony also resulted in complete interruption of the cycle. Some first-generation schizonts were still apparent 24 hr after treatment and a few second-generation schizonts were able to develop; however, most of these degenerated rapidly and exhibited incomplete merogony (Fig. 5). The degenerative changes observed in second-generation schizonts were identical to those described for first-generation schizonts. Because no further stages were noted in any of the treated animals, it is assumed that all second-generation schizonts degenerated. No endogenous stages or oocysts were detected in the caecal content at days 8, 10, and 12 after treatment.

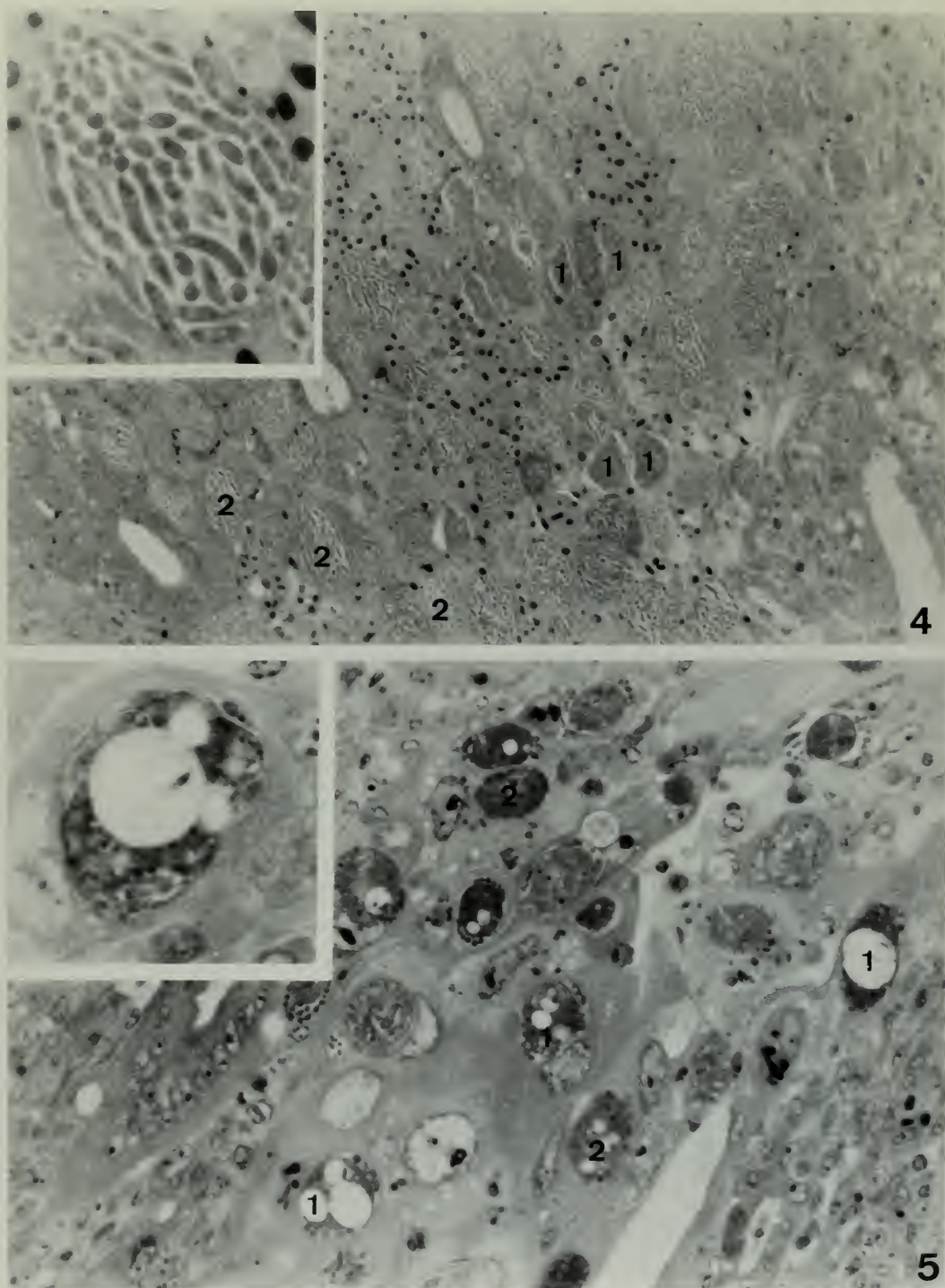
Treatment on day 3 (group 3) or at the stage of first and second schizogony also resulted in complete interruption of the cycle. First-generation schizonts were no longer present but second-generation schizonts were numerous. On the first day after treatment, some of the schizonts had already started to degenerate and nearly all had degenerated by the third day after treatment. In 1 animal, some degenerating gametocytes could be detected on day 7 (i.e., 4 days after treatment). Oocysts were never observed, either histologically or coprologically.

Treatment on day 4 (group 4) or during second schizogony and gametogony resulted in complete degeneration of these stages within 3 days and in a complete interruption of the life cycle. Degeneration of gametocytes was characterized by a ballooning appearance and loss of internal

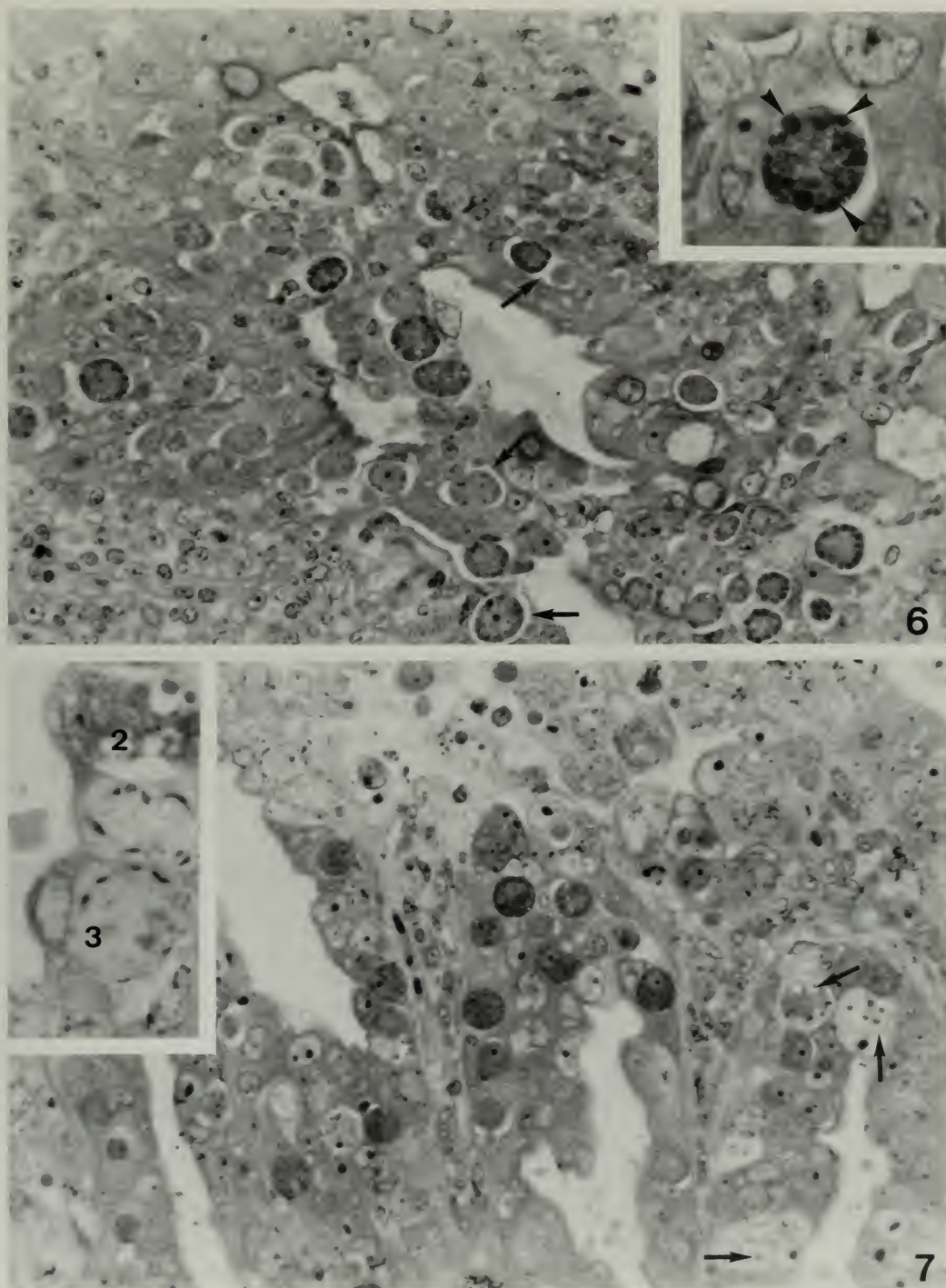


FIGURES 1-3. 1. Caecal crypt epithelium of untreated chicken (day 1 postinfection). Toluidine blue,  $\times 320$ . Trophozoite in parasitophorous vacuole (arrow). 2. Caecal crypt epithelium of untreated chicken (day 3 postinfection). Toluidine blue,  $\times 320$ . Multinucleated first-generation schizont in enterocyte of the crypt (arrow). 3. Caecal crypt epithelium of diclazuril-treated chicken (day 3 postinfection). Toluidine blue,  $\times 320$ . Degenerated first-generation schizont: intraprotoplasmic vacuoles (arrows) and indistinct internal structures.





FIGURES 4, 5. 4. Caecal wall of untreated chicken (day 5 postinfection). Toluidine blue,  $\times 128$  (insert  $\times 320$ ). Second-generation schizonts in lamina propria: immature schizont (1); mature schizonts (2) with numerous merozoites (insert). 5. Caecal wall of diclazuril-treated chicken (day 6 postinfection). Toluidine blue,  $\times 128$  (insert  $\times 320$ ). Degenerating second-generation schizonts: intraprotoplasmic vacuoles (1); incomplete merogony (2). Merozoites are sometimes present in the intraprotoplasmic vacuoles (insert).



FIGURES 6, 7. 6. Caecal epithelium of untreated chicken (day 6 postinfection). Toluidine blue,  $\times 128$  (insert  $\times 320$ ). Macrogametocytes in villus epithelium: well-defined parasitophorous vacuole (arrow); typical wall-forming bodies (arrowheads—insert). 7. Caecal epithelium of diclazuril-treated chicken (day 6 postinfection). Toluidine blue,  $\times 128$  (insert  $\times 320$ ). Degenerated gametocytes with a ballooned aspect and many intraprotoplasmic vacuoles (arrows). Macrogametocyte (2) and microgametocyte (3) in detail.



TABLE I. *Lesion scores at autopsy.*

Group	Lesions* at days postinfection											
	0	1	2	3	4	5	6	7	8	9	10	12
1	†	‡	nd	nd	0	0			0	0	0	0
2	†	‡	nd	0	0.5	0			0	0	0	0
3	†		‡	1.5	2.5	1.5	1.0	2.5		0	0	0
4	†			‡	4.0	2.0	1.0	1.0	1.5	1.0	0	0
5	†				‡	1.5	2.0	1.0	1.0	0.5	0.5	0.5
6	†					‡	2.0	1.5	1.0	0.5	1.0	1.0
7	†						‡	1.0	1.5	0.5	0.5	0.5
8§	†	nd	nd	nd	0	0	0	0	0	0	0	0
9	†	nd	nd	nd	4.0	4.0	4.0	3.0	2.0	2.0	2.0	2.0

\* Lesion scores (average of 2 animals) (according to Johnson and Reid, 1970); nd, not done.

† Artificial infection with  $5 \times 10^5$  sporulated oocysts of *E. tenella*.

‡ Single oral treatment with diclazuril at 5 mg/kg.

§ Diclazuril at 1 ppm in the feed starting from day -1 until day 12.

structure. The size of degenerating gamonts was approximately twice as large as normal gamonts. The cytoplasm became more translucent and the wall-forming bodies disappeared. In addition, gamonts developed in a very reduced parasitophorous vacuole compared to parasites in the infected, nonmedicated control birds (Fig. 7).

Treatment on day 5 (group 5) produced results comparable to those of group 4. Degenerating schizonts and micro- and macrogametocytes were seen up to 2 days after treatment. The cycle did not reach patency as no oocysts were formed.

Treatment on day 6 (group 6) or on day 7 (group 7) or at the moment of an established patent infection resulted only in degeneration of the remaining second-generation schizonts and of the gametocytes. Apparently, there was no effect on the oocysts as they remained present in the caecal content until the end of the experiment on day 12.

The histological findings in the birds treated with 1 ppm in the feed (group 8) were similar to those of group 1. A few first-generation schizonts were observed at days 1 and 2, but they rapidly degenerated. No intestinal stages were found from day 4 onward, and the cycle was completely interrupted before second-generation schizonts could develop.

#### Lesion development in treated and nontreated animals

The average scores of the macroscopic lesions are presented in Table I. All of the control animals (group 9) developed severe caecal lesions, characterized by a haemorrhagic appearance of the intestinal wall and haemorrhagic contents. In

the recovery phase, most animals developed a solid caecal core. Toward the end of the experiment, recovery was almost complete, but a few lesions were still present. The clinical picture in the treated animals was markedly different. The animals of groups 1, 2, and 8 developed no lesions. However, the animals of groups 3-7 all developed lesions that were of nearly comparable severity as the infected, nonmedicated control animals. The lesions, however, tended to disappear more quickly in the treated animals.

At the microscopic level, caecal lesions were characterized by profound destruction of the epithelial layer, thickening of the caecal wall, and the presence of numerous small haemorrhages and inflammatory cells (heterophils and round cells) in the lamina propria and submucosa. In control animals, lesions were present from day 4 until day 12. Haemorrhages were particularly obvious on days 4-6 or during the second schizogony. In treated animals, the presence and severity of the lesions depended on the timing of the treatment; hardly any lesions developed when treatment was given before second-generation schizonts appeared. Treatment later in the life cycle had only a minor effect on the severity of the lesions, but slightly faster healing of the intestinal wall usually was observed.

#### DISCUSSION

The site of action of diclazuril on the endogenous stages of *E. tenella* was investigated clinically and morphologically in experimentally infected chickens. For this purpose, the basic experimental design of administering medication for an abbreviated period, coinciding with the presence of well-defined developmental stages of the parasite, was used. Studies of similar design have been performed with other anticoccidials (Ryley, 1967; Ryley et al., 1974; McQuiston and McDougald, 1981). Medication in these studies usually was administered through the feed at the proposed dose level for a restricted period. This approach was not used in the present study because we searched for distinct peak anticoccidial activities. Furthermore, medication in the feed has the disadvantage that it may take some time before effective drug levels are achieved. The variable uptake of feed between individual animals may further complicate interpretations of the data. For this reason, administration of a single high oral dose was preferred, to enhance the chances of finding peak-related anticoccidial effects, rather than dose- or time-related effects.

Diclazuril was found to have activity against all developmental stages of *E. tenella*. The most striking morphological effect was the rapid and extensive degeneration of the endogenous stages shortly after treatment, which is an indication that diclazuril was lethal.

The efficacy of diclazuril on trophozoites could not be established histologically because trophozoites were not found in the treated animals. Although a substantial reduction in the number of first-generation schizonts was noted 24 hr after treatment (Table I, group 1), this could either be the consequence of direct activity of the drug on the trophozoite or be the result of residual activity of the drug against developing schizonts. The answer can only be provided by *in vitro* studies.

The effect of diclazuril on first- and second-generation schizonts was complete, as animals treated during schizogony (groups 2 and 3) never reached a patent cycle. After treatment, schizonts rapidly degenerated and few merozoites differentiated. It is obvious that the clinical picture (caecal lesion scores) was not affected by treatment once the second-generation schizonts had started to invade the lamina propria and submucosa. Nevertheless, recovery in treated animals was faster than in the infected, nontreated controls, an argument in favour of a complete lethal effect on the schizonts.

There were clear indications that diclazuril is also fully lethal against the sexual stages because most gamonts showed degeneration shortly after treatment. Animals treated on day 5 or at the peak of gametogony did not excrete oocysts, which is an indication that all gamonts present at that time did not develop further and that they degenerated. All animals treated at day 6 or later passed oocysts until the end of the experiment. These animals, however, already had a patent infection at the time of treatment and it was to be expected that oocysts would be found in the caecal content. The reason why excretion of oocysts persisted for so long after treatment may be related to the slow emptying of the caeca, of which the normal physiological function had been severely affected by the infection.

The histological findings in the animals treated with 1 ppm in the feed or with a single 5-mg/kg oral dose 1 day after infection were fully comparable and resulted in complete interruption of the life cycle and total absence of coccidiosis-related lesions. In view of the site of action of diclazuril against *E. tenella*, this is certainly not

surprising because the drug is already fully effective before the lesion-inducing second-generation schizonts appear. The lethality of diclazuril explains the complete absence of oocysts in the feces of treated animals. This makes diclazuril a valuable compound for the prophylactic treatment of coccidiosis.

Diclazuril is a nucleotide analogue. Up to now, very few anticoccidials of this type have been developed or marketed. Pyrimidine derivatives include an azauracil derivative (Ryley et al., 1974), and aryltriazine (Chappel et al., 1974), and triazinones (Mehlhorn et al., 1984). Arprinocid (McManus et al., 1980; Ball et al., 1985), eniptycine riboside (Kobayashi et al., 1986), and an aminopurine (Matsuno, 1986) are purine analogues. In general, all of these nucleotide anticoccidials are considered to be lethal and to affect nearly all endogenous developmental stages. The susceptibility of these stages varies somewhat within and among the different *Eimeria* species.

Given the typical efficacy profile of diclazuril against the different *Eimeria* species in the chicken and because it is well known that different species respond differently to a particular anticoccidial, further investigations on the activity of diclazuril on the endogenous stages of other poultry coccidia species, such as *E. necatrix*, *E. brunetti*, *E. maxima*, *E. acervulina*, and *E. mivati*, are warranted.

#### ACKNOWLEDGMENTS

The skillful technical help of J. Fransen and L. Leijssen in preparing the photographs and the secretarial work of J. Frederickx and A. Wuyts are gratefully acknowledged.

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## IN VIVO ACTION OF THE ANTICOCIDIAL DICLAZURIL (CLINACOX®) ON THE DEVELOPMENTAL STAGES OF *EIMERIA TENELLA*: AN ULTRASTRUCTURAL EVALUATION

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**ABSTRACT:** A single 5-mg/kg oral dose of diclazuril affected both the asexual and sexual development of *Eimeria tenella* in experimentally inoculated chickens. In second-generation schizonts, early growth and nuclear divisions progressed normally, but a marked inhibition of merozoite formation was observed. Exogenesis of merozoites was largely prevented, whereas production of micronemes, amylopectin granules, and dense bodies and the formation of rhoptries, conoid, and pellicle continued. All these subcellular organelles accumulated, together with differentiated nuclei, within the main cytoplasmic mass. In the end, complete necrosis of the schizonts occurred. In macrogamonts, dilation of the rough endoplasmic reticulum around type II wall-forming bodies, fusion of type II wall-forming body contents, disturbance of the normal parallel arrangement of rough endoplasmic reticulum, and disruption of row formation of amylopectin granules became evident. In the microgamonts, normal evagination of microgametes was prevented; the flagellar complex formed within the main cytoplasmic mass and the differentiated nuclei remained present within the parasite body. The macro- and microgamonts also ended up in a stage of complete necrosis. These data indicate that diclazuril treatment primarily affects the normal differentiation of the respective endogenous stages during parasite development. This leads to complete degeneration of schizonts and gamonts indicating the lethal effect of this new anticoccidial compound.

The benzeneacetonitrile, diclazuril, is a recently introduced anticoccidial belonging to the nucleoside analogue group. Dose-titration studies and pilot floor-pen trials clearly demonstrated its potential as an effective agent against all major pathogenic *Eimeria* spp. in chickens (Vanparijs et al., 1988). In *E. tenella*, the absence of clinical lesions in treated birds suggested that diclazuril acts against the asexual stages of parasite development. To explore this further and to see whether diclazuril was also active against sexual stages, a laboratory study was performed in which diclazuril was given curatively as a single oral dose at various time intervals after experimental inoculation. In this way, we intended to obtain information about the mode of action of this anticoccidial. The coprological, clinical, and histological findings of this study are described in the accompanying paper (Maes et al., 1988). These data showed that oocyst production was completely inhibited when chickens were given a single oral dose of 5 mg/kg during the development of both the first- and the second-generation schizonts. Diclazuril treatment before or during first-generation schizogony resulted in the near absence of caecal hemorrhagic lesions, in-

dicating that second-generation schizonts either did not develop or were only present in very small numbers. The most obvious histological change in developing schizonts of both generations was inhibition of merozoite differentiation and budding, which finally led to complete degeneration of the parasites. Treatment during gametogony resulted in the degeneration of developing gamonts and a clear inhibition of oocyst production. All these degenerative changes were indicative of the lethal effects of this drug.

In this article, we describe the ultrastructural changes in the various endogenous stages of *E. tenella* from diclazuril-treated chickens. These micromorphological changes provided more detailed information regarding the mechanism of action of this new anticoccidial drug.

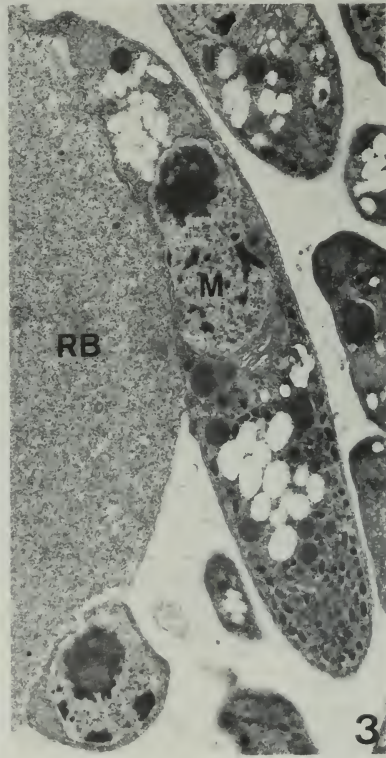
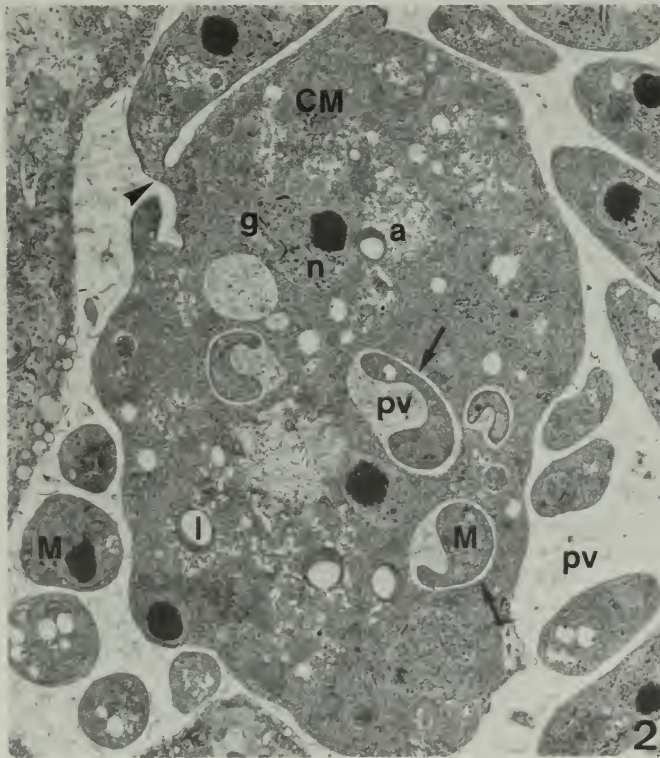
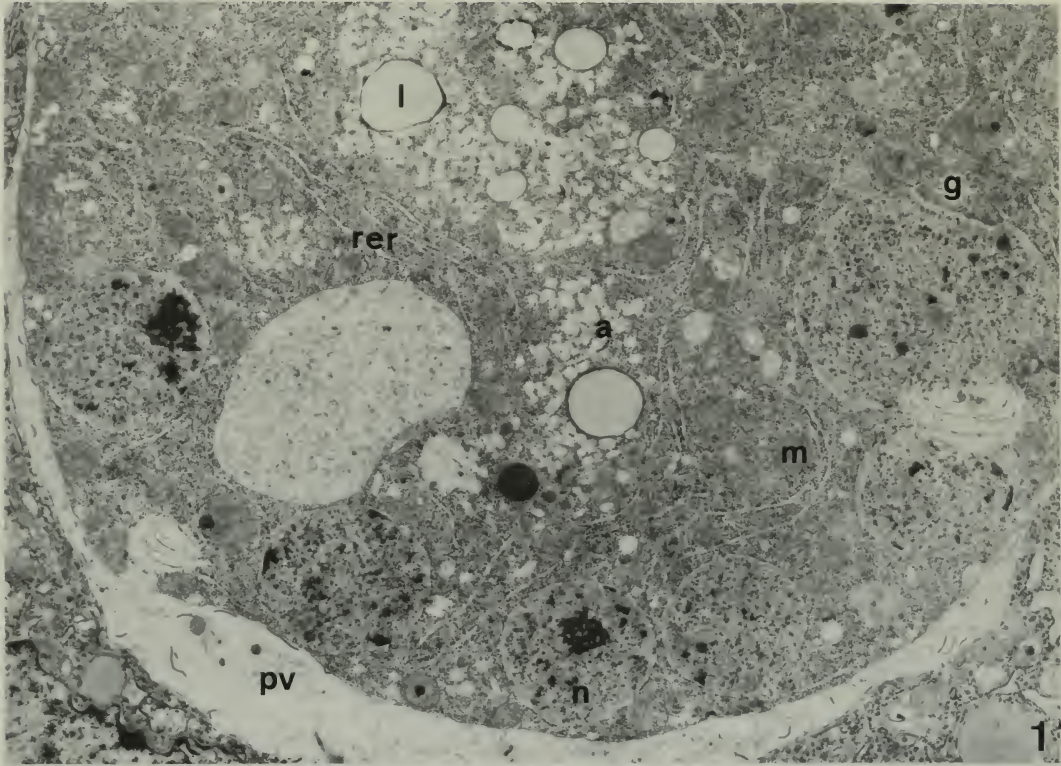
### MATERIALS AND METHODS

Tissue samples were taken from the caeca of the same chickens as were used for coprological, clinical, and histological examination (Maes et al., 1988). Hisex chickens were artificially inoculated with 50,000 oocysts of *E. tenella* at day 0. A single oral dose of 5 mg/kg diclazuril was given at either 1, 2, 3, 4, 5, 6, or 7 days after inoculation. The chickens were necropsied at 24, 48, and 72 hr after treatment and further at 8, 10, and 12 days after inoculation. Small tissue samples of the caeca were immersed in a mixture of 2% paraformaldehyde + 2.5% glutaraldehyde (in phosphate buffer, pH 7.4) for 3-14 days. After thorough rinsing at 4°C in several changes of the phosphate buffer supplemented with 7.5% sucrose, the samples were postfixed

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for 1 hr at 4 C in 2% OsO<sub>4</sub> in 0.05 M veronal acetate buffer (pH 7.4) containing 3.2% sucrose. After a 5-min rinse in 0.05 M veronal acetate buffer supplemented with 7% sucrose (pH 7.4, 4 C), the tissue was impregnated with 0.5% uranyl acetate in 0.05 M veronal acetate buffer (pH 5.2) for 40 min at 4 C, briefly rinsed, dehydrated in a graded series of ethanol, and embedded in Epon. Two- $\mu$ m-thick sections were prepared and stained with toluidine blue for topographical localization of the parasites. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM 410 electron microscope.

## RESULTS

### The asexual stages

Because first-generation schizonts were only rarely observed, particularly after treatment, we limit our descriptions to the ultrastructure of the developing second-generation schizonts. No difference was noted between schizonts developing at different time intervals after inoculation, either in treated or in untreated chickens. Consequently, distinctions between schizonts fixed at different times postinoculation will not be made.

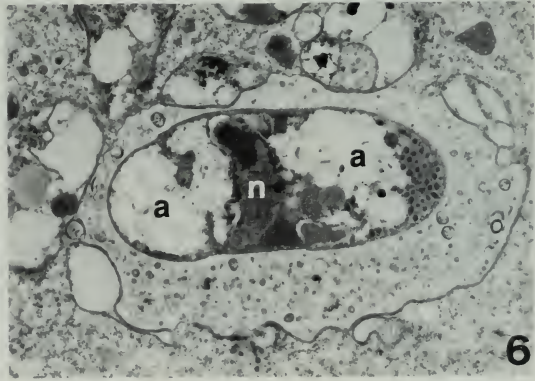
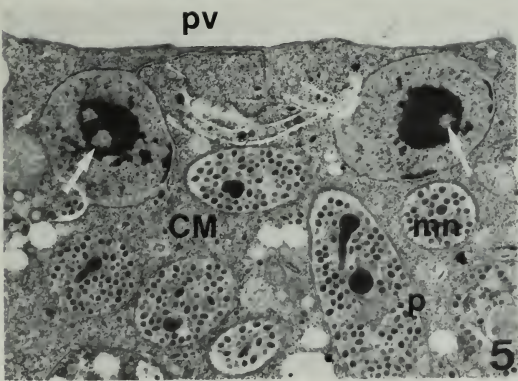
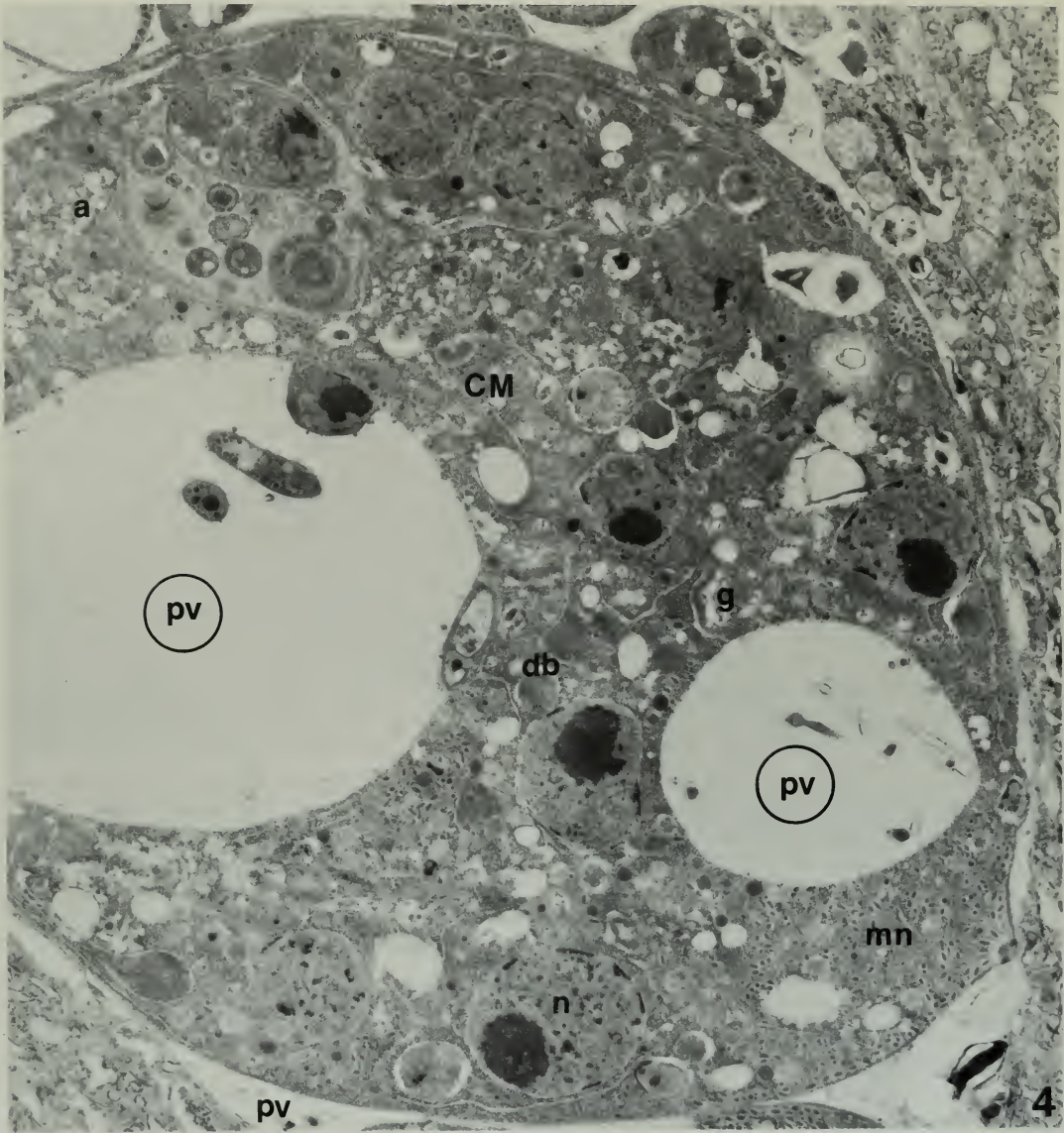
One day after treatment, the early uninucleate second-generation schizonts and schizonts undergoing growth and nuclear division were normal in appearance. Some dilation of the nuclear envelope and rough endoplasmic reticulum cisternae was evident, but was comparable to that found in the schizonts from untreated chickens. In the early stage of differentiation, schizonts in which the nuclei had moved to the periphery contained numerous amylopectin granules in the main cytoplasmic mass (Fig. 1). No other ultrastructural changes were apparent. Comparable changes were seen in schizonts with budding and free merozoites (Fig. 2). Additionally, in some schizonts micronemes as well as some differentiated nuclei were observed within the main cytoplasmic mass. Differentiated nuclei were char-

acterized by the close association of most of the chromatin with the inner nuclear membrane and by the compactness of the nucleolus. Several fully differentiated merozoites were not separated from the main cytoplasmic mass (Fig. 3). This phenomenon was never seen in untreated control chickens at this stage of schizont differentiation. At the relatively large areas of mutual contact, merozoites and residual bodies shared plasma membranes. At this site, the merozoite showed incomplete pellicle formation. Intercytoplasmic contacts without interjacent plasma membrane were also seen between both schizont parts. Some vacuoles within the main cytoplasmic mass contained merozoites (Fig. 2). No changes in ultrastructure were noted in nucleus, nucleolus, mitochondria, rough endoplasmic reticulum, and free ribosomes. Dilation of rough endoplasmic reticulum and nuclear envelope was not apparent.

Two or 3 days after diclazuril treatment, the number of early schizonts decreased drastically. Only a few parasites with multiplying, randomly distributed nuclei were present. These appeared normal. Once the nuclei had moved to the periphery of the schizont cytoplasm, amylopectin formation was evident. Some early schizonts were in early stages of degeneration while others were completely necrotic. At later times after treatment, most of the early schizonts were in advanced stages of degeneration or could no longer be found. The formation of free merozoites was largely inhibited, whereas large numbers of differentiated nuclei remained present within the main cytoplasmic mass. Production of micronemes, amylopectin granules, rhoptries, dense bodies, and the pellicular structures went on in the free merozoites as well as in the main cytoplasmic mass. These masses thus showed clear accumulation of these subcellular organelles (Figs.

FIGURES 1–3. Ultrastructural changes in second-generation schizonts 1 day after diclazuril treatment. 1. Treated on day 3 and sacrificed on day 4 after inoculation. Early second-generation schizont in which the nuclei have already moved to the periphery. The formation of amylopectin granules in the main cytoplasmic mass is abnormal ( $\times 40,800$ ). 2. Chicken treated on day 3 and sacrificed on day 4 after inoculation. Schizont-forming merozoites. The main cytoplasmic mass contains abnormal accumulation of amylopectin granules and some differentiated nuclei. Some merozoites are present in large vacuoles within the main cytoplasmic mass (arrows). The small area of intercytoplasmic contact between the budding merozoite and the cytoplasmic mass (arrowhead) is normal in this schizont ( $\times 6,450$ ). 3. Chicken treated on day 4 and sacrificed on day 5 after inoculation. The relatively large area of mutual contact between a fully differentiated merozoite and the residual body is abnormal ( $\times 11,730$ ). Abbreviations used in the figures: a: amylopectin granules; bb: basal body; CM: main cytoplasmic mass; db: dense bodies; f: flagella; g: Golgi field; HC: host cell; l: lipid globule; lg: light-dense granules; M: free merozoite; m: mitochondria; MG: microgamete; mn: micronemes; n: nucleus; nu: nucleolus; ow: oocyst wall; p: pellicle; pv: parasitophorous vacuole; r: free ribosomes; RB: residual body; rer: rough endoplasmic reticulum; I: wall-forming body type I; II: wall-forming body type II.





4, 5). For comparison, the normal morphology of a mature schizont with many free merozoites and small residual bodies, as observed in untreated control chickens, is shown in Figures 7 and 8. In the diclazuril-treated chickens, the large vacuoles seen in the main cytoplasmic mass of many schizonts represented transsections of the infolded parasitophorous vacuole wherein merozoite formation could be observed (Fig. 4). On 2 but particularly 3 days after treatment, many nuclei present in merozoites as well as the main cytoplasmic mass showed abnormal intranucleolar clarifications (Fig. 5). Fusion of amylopectin granules was often seen in late schizogony, in merozoites (Fig. 6) as well as in the main cytoplasmic mass. Complete necrosis with extensive vacuolation of schizonts was observed at 2 days but was most pronounced from 3 days after drug administration onward (Fig. 9). The necrotic parasites were phagocytized by macrophages and further degraded.

#### The sexual stages

*Development of macrogamonts in diclazuril-treated chickens:* As early as 1 day after treatment, a slight to sometimes pronounced dilation of the rough endoplasmic reticulum was observed around slightly swollen type II wall-forming bodies (WFBII) in several immature macrogamonts (Fig. 10). WFBII in some macrogamonts were fused (Fig. 11), while others presented normal WFBII. A pronounced accumulation of light-dense granules and small bodies (resembling small type I wall-forming bodies, WFBI) close to the outer plasma membrane was observed in some gamonts. In most macrogamonts, parallel arrangement of the rough endoplasmic reticulum cisternae with interspersed rows of amylopectin granules, as seen in untreated control chickens (Fig. 10), was not observed in diclazuril-treated birds (Figs. 11, 12). Other subcellular organelles remained unaffected. Formation of the various kinds of bodies and granules was not interrupted during differentiation.

Moderate to pronounced cytoplasmic swelling up to complete necrosis was seen in several mature gamonts, before as well as during wall formation. Membranes of the rough endoplasmic reticulum as well as those surrounding the WFBII were disintegrating and the ribosomes clustered together (Fig. 13). Degenerating parasites were closely surrounded by the host cell cytoplasm so that the parasitophorous vacuole was indistinct. This degeneration became more frequent 2 days after treatment and finally affected all macrogamonts. The degenerated gamonts in a stage of oocyst wall development showed abnormal, irregular wall formation (Fig. 14).

*Development of microgamonts in diclazuril-treated chickens:* The growth phase, where the microgamonts increased in size and where nuclear divisions occurred, was not affected. As differentiation of microgamonts began, the nuclei moved normally toward the periphery of the main cytoplasmic mass and chromatin condensation occurred. The flagellar complex developed in the main cytoplasmic mass but did not protrude into the parasitophorous vacuole (Fig. 15). The condensed chromatin portion of the nuclei separated from the pale portion but remained present within the main cytoplasmic mass (Fig. 16). Fusion of accumulated amylopectin granules was obvious. Complete degeneration of the microgamonts was seen mostly within 1–2 days after treatment.

#### Host tissue

At no time after treatment were there any morphological changes in the cells of the mucosa or lamina propria of the chickens. This is at variance with the ultrastructure of comparable cells in untreated control chickens observed.

#### DISCUSSION

A single 5-mg/kg dose of diclazuril induced marked ultrastructural changes in schizonts as well as in macro- and microgamonts of *E. te-*

FIGURES 4–6. Ultrastructural changes in second-generation schizonts 2–3 days after diclazuril treatment. 4. Chicken treated on day 4 and sacrificed on day 6 after infection. The formation of free merozoites is largely inhibited. Many differentiated nuclei remain distributed in the main cytoplasmic mass wherein accumulation of amylopectin, micronemes, and dense bodies is obvious. Note the abnormal transsections of the parasitophorous vacuole (encircled) in the main cytoplasmic mass ( $\times 8,600$ ). 5. Chicken treated on day 3 and sacrificed on day 6 after inoculation. Part of the main cytoplasmic mass. The intranucleolar clarifications (arrows) are abnormal. Intracytoplasmic pellicle and rhoptry formation and large numbers of micronemes are seen ( $\times 9,030$ ). 6. Chicken treated on day 3 and sacrificed on day 5 after inoculation. Fusion of amylopectin granules with deformation of the nucleus in a free merozoite ( $\times 8,170$ ).



*nella*. These changes resulted in complete degeneration of the endogenous stages.

The first detectable ultrastructural alteration in developing second-generation schizonts was the formation of amylopectin granules in the main cytoplasmic mass of immature schizonts and maturing schizonts with budding merozoites. Accumulation of amylopectin granules was never observed in comparative schizonts from untreated control chickens. No differences from normal morphology were seen in schizonts during stages of early growth and nuclear division. Later on, a clear inhibition of merozoite exogenesis, indicated by abnormal or near absence of budding, was obvious. This was also evident by light microscopy (Maes et al., 1988). Ultrastructural observations further showed that the affected schizonts had differentiated nuclei and micronemes, amylopectin granules, rhoptries, etc., formed and accumulated within the main cytoplasmic mass. In control tissue, these subcellular organelles were only observed in budding and free merozoites, as has been described previously (McLaren and Paget, 1968; Long, 1971; Hoppe, 1976; Chobotar and Scholtyseck, 1982; Mehlhorn et al., 1984). The large vacuoles seen within the main cytoplasmic mass after diclazuril treatment corresponded to the large-sized vacuoles observed by light microscopy in histological sec-

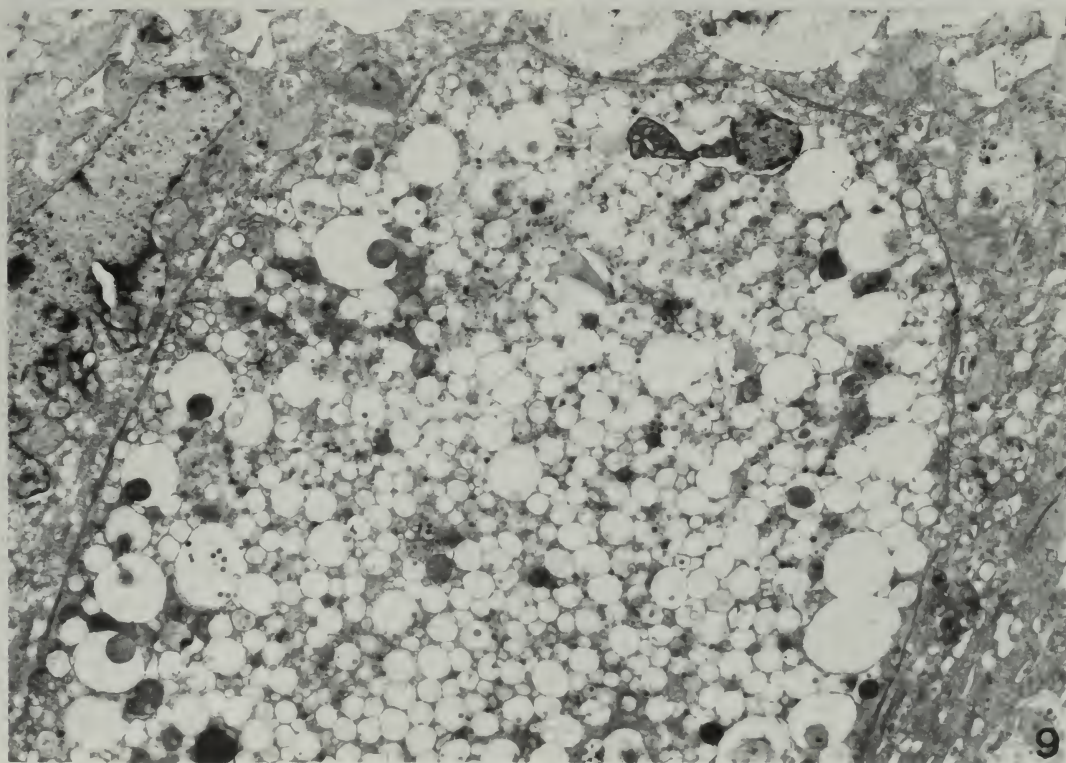
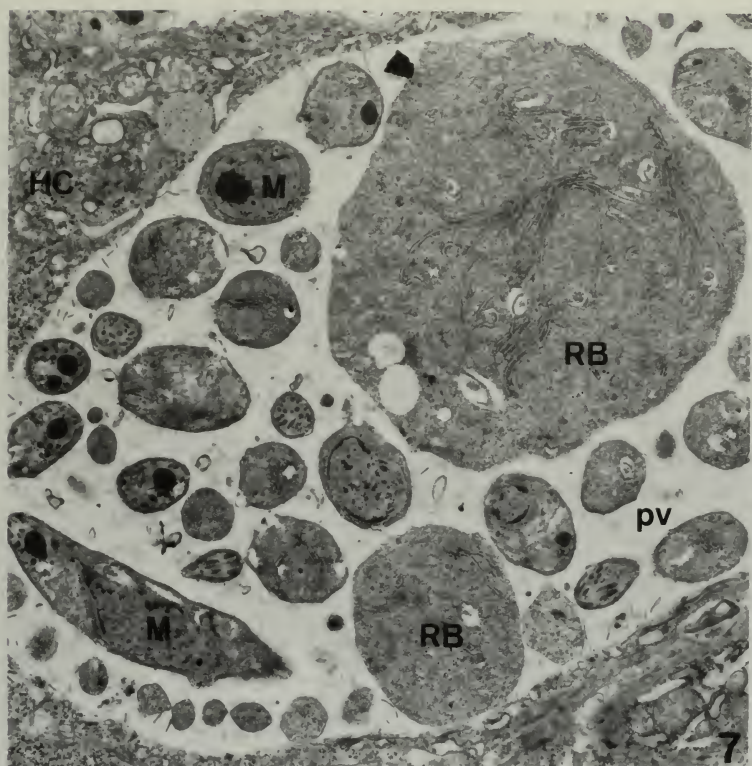
tions (Maes et al., 1988). These vacuoles represented abnormal invaginations of the parasitophorous vacuole. Such large vacuoles, containing occasional free merozoites, were also observed after arprinocid treatment (Ball et al., 1985). These abnormal invaginations may represent extensions of the available outer surface for exogony. Thus, the observations indicated that diclazuril did not affect early growth and nuclear divisions but clearly induced abnormal differentiation. Abnormal differentiation was also seen in some first-generation schizonts (not described). Failure to complete development finally led to fusion of amylopectin granules and degeneration and lysis of accumulated organelles resulting in the observed necrosis of schizonts. The fusion of amylopectin granules likely contributed to the apparent vacuolization of parasites observed light microscopically (Maes et al., 1988).

Merozoite formation was also disturbed by related nucleoside analogues such as azauracil derivatives (Ryley et al., 1974), triazinones (Mehlhorn et al., 1984), and the benzylpurine arprinocid (Ball et al., 1985). Similar changes were also described after application of robenidine (Ryley and Wilson, 1971; Lee and Millard, 1972). After treatment with azauracil derivatives and triazinones, all organelles of merozoites such as conoid, pellicle, and micronemes were also found to

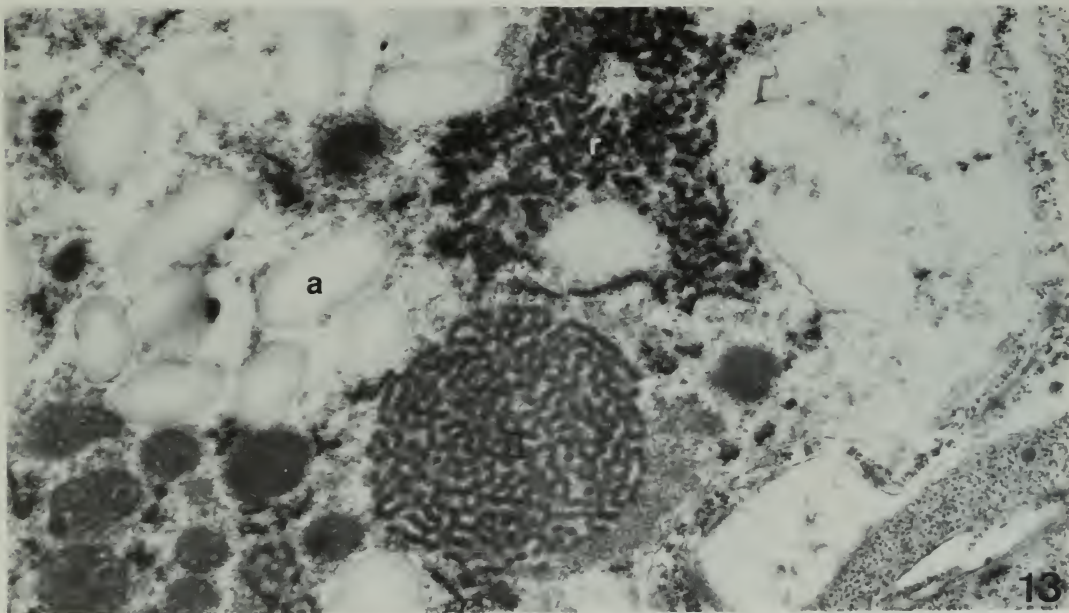
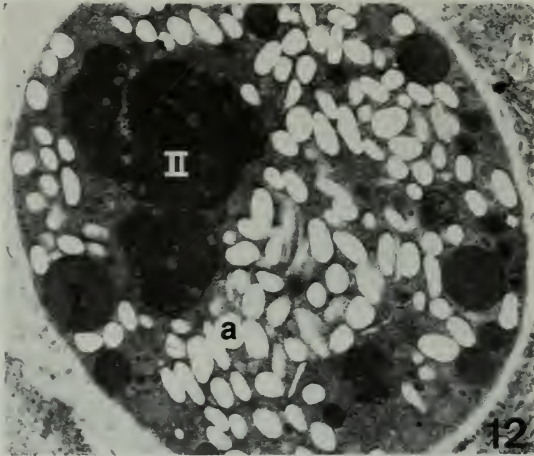
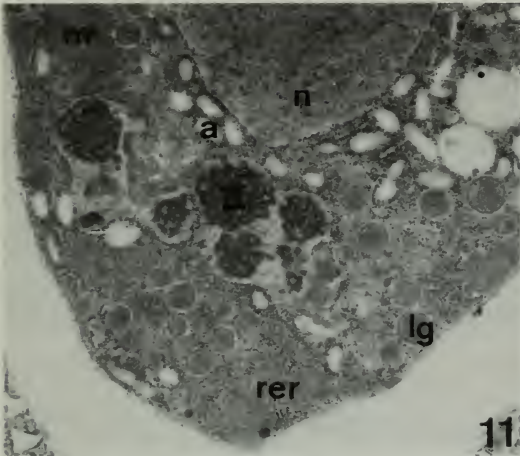
FIGURES 7-9. 7. Untreated control chicken, day 4 after inoculation. Survey of a near fully developed second-generation schizont. Free merozoites and some small residual bodies (lacking merozoite-specific subcellular organelles) can be observed ( $\times 7,310$ ). 8. Untreated control chicken, day 4 after inoculation. Normal ultrastructure of free merozoites in the parasitophorous vacuole. Note the individual amylopectin granules ( $\times 12,740$ ). 9. Chicken treated with diclazuril on day 4 and sacrificed on day 6 after inoculation. Severe vacuolization indicating complete necrosis of schizont of the second generation ( $\times 7,310$ ).

FIGURES 10-13. Developing macrogamonts in untreated and diclazuril-treated chickens. 10. Developing macrogamonts in untreated chicken. Day 5 after inoculation. Presence of WFB I, WFB II with surrounding rough endoplasmic reticulum, and light-dense granules. The rows of amylopectin granules, close to and in between the rough endoplasmic reticulum cisternae, are arranged in parallel ( $\times 19,550$ ). 11. Chicken treated on day 6 and sacrificed on day 7 after inoculation. Dilation of the rough endoplasmic reticulum around the WFB II and initiation of fusion. Amylopectin is formed but not in rows and the rough endoplasmic reticulum is randomly distributed within the cytoplasm ( $\times 13,800$ ). 12. Chicken treated on day 5 and sacrificed on day 6 after inoculation. Fusion of WFB II. Randomly distributed rough endoplasmic reticulum and amylopectin granules ( $\times 8,190$ ). 13. Chicken treated on day 5 and sacrificed on day 6 after inoculation. Part of a degenerating macrogamont. The membranes of the rough endoplasmic reticulum are disintegrated and clustering of the ribosomes is apparent ( $\times 26,100$ ).

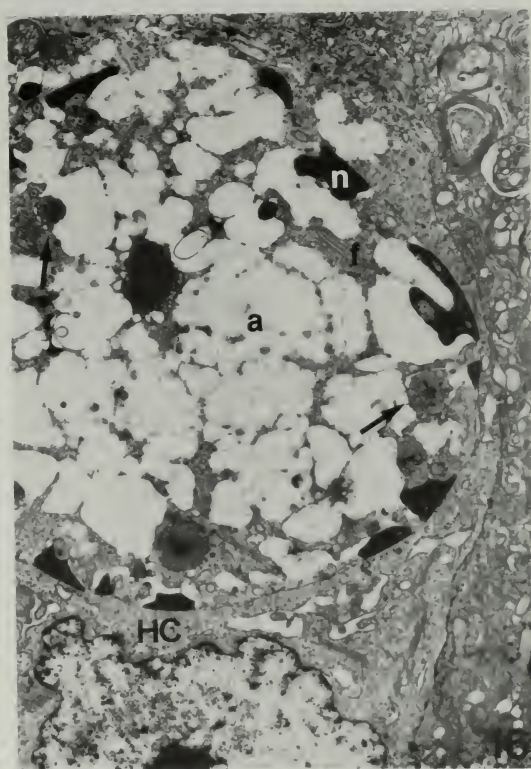
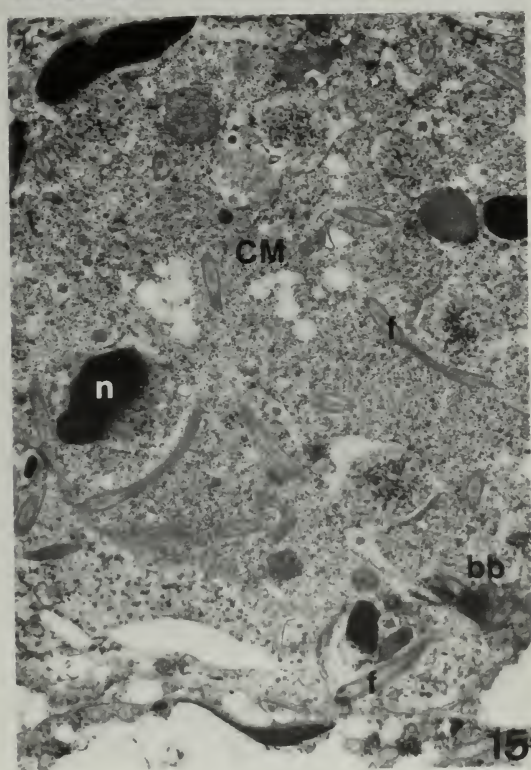
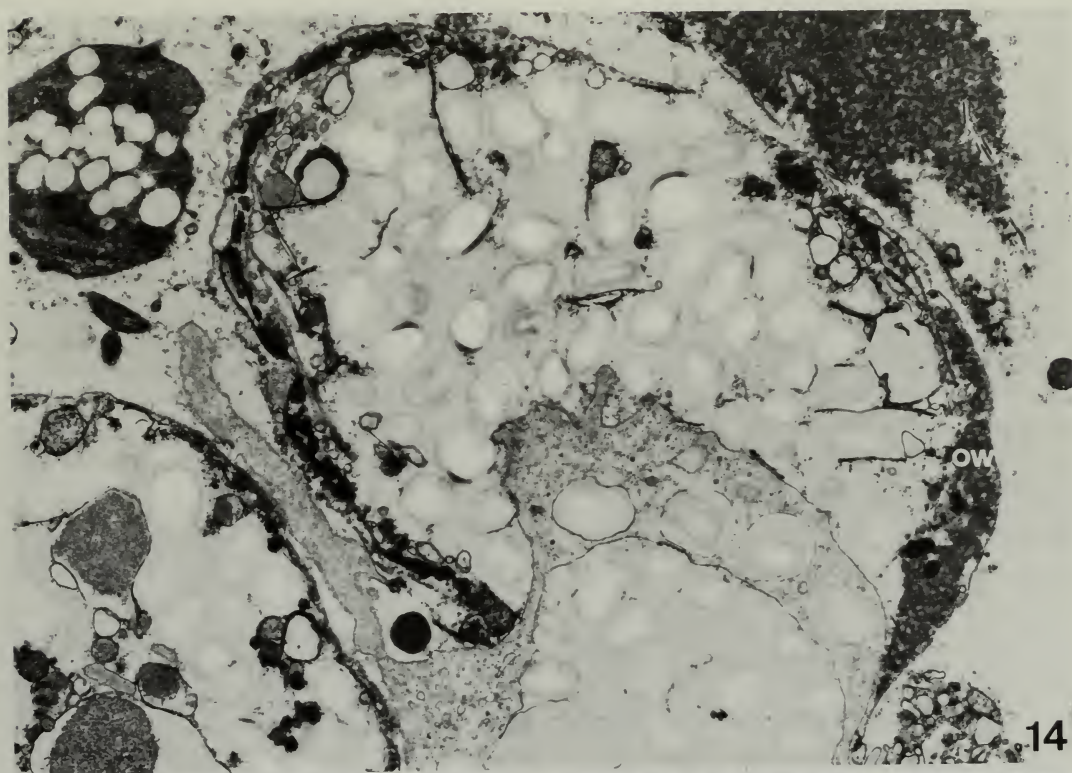
FIGURES 14-16. 14. Chicken treated on day 5 and sacrificed on day 7 after inoculation. Necrotic, swollen zygote with irregular oocyst wall formation ( $\times 9,720$ ). 15, 16. Differentiating microgamonts in diclazuril-treated chickens. 15. Chicken treated on day 5 and sacrificed on day 6 after inoculation. Flagella are formed within the main cytoplasmic mass and do not protrude into the parasitophorous vacuole, as normally occurs in untreated control chickens ( $\times 10,350$ ). 16. Chicken treated on day 7 and sacrificed on day 8 after inoculation. The condensed chromatin portion of the nuclei and flagella remain present within the main cytoplasm. Fusion of large numbers of amylopectin granules is obvious. The parasite, which is closely surrounded by a host cell, has started to degenerate. Arrows indicate pale portions of the nuclei ( $\times 6,480$ ).













accumulate in the cytoplasmic mass (Ryley et al., 1974; Mehlhorn et al., 1984). This was not described for the polyether ionophorous antibiotics and arprinocid (Mehlhorn et al., 1983; Ball et al., 1985). The near absence of effect of antibiotics on intracellular stages was suggested to be due to the incapability of these drugs to penetrate intact host cells (Mehlhorn et al., 1983). Apparently, this was not the case for diclazuril and related compounds. In schizonts, complete degeneration after treatment with most of the anticoccidial compounds was preceded by extensive swelling of the perinuclear space and the endoplasmic reticulum. This swelling was claimed to disturb merozoite formation. Wang and co-workers (1981) suggested that altered P-450-mediated microsomal metabolism by arprinocid-1-N-oxide could cause destruction of the endoplasmic reticulum and lead to cell death. In the present study, dilation of rough endoplasmic reticulum and the nuclear envelope was sometimes seen in early schizonts and microgamonts, but was observed with the same frequency in controls. Because this dilation was always seen in parasites with a clear cytosol surrounded by less well-preserved host tissue, localized areas of inadequate fixation may be responsible for the dilation. Moreover, dilation of endoplasmic reticulum is not always found to be a sign of cell degeneration (Thomopoulos, 1987). Therefore, swelling of endoplasmic reticulum probably is not a prerequisite for parasite degeneration induced by diclazuril treatment.

Polyether antibiotics act very early in asexual development. Diclazuril, however, not only affects the different asexual stages but also the sexual stages of parasite development as has already been shown by Maes et al. (1988). In macrogamonts, the ultrastructural morphology of the WFBII was changed (dilation of surrounding rough endoplasmic reticulum, fusion of contents of different individual bodies) and the normal parallel arrangement of rough endoplasmic reticulum cisternae and the row formation of amylopectin granules was disturbed. These changes may also be suggestive of aberrant differentiation that may lead to the observed degeneration of macrogamonts. Dilation of rough endoplasmic reticulum and ensuing malformation of the WFBII were also observed in macrogamonts of *E. tenella* and *E. acervulina* after treatment with triazinones (Mehlhorn et al., 1984) and of *E. maxima* after treatment with arprinocid (Pittilo et al., 1981).

According to Pittilo et al. (1981) and Mehlhorn et al. (1984), these changes led to abnormal wall formation and therefore to necrosis. In this study, necrosis occurred both before and during oocyst wall formation, which indicates that abnormal wall formation may be a secondary degenerative change. In diclazuril-treated chickens, the development of microgamonts proceeded normally up to the formation of the flagellar complex. In untreated control animals, this complex evaginated into the parasitophorous vacuole from peripherally localized protrusions. The condensed part of the nucleus was then incorporated into the evaginating protrusion and the flagella with associated nucleus was finally split off to form the microgamete (McLaren, 1969; Chobotar and Scholtyseck, 1982; Mehlhorn et al., 1984). In treated chickens, the flagellar complex and condensed nuclei were formed but failed to evaginate into the parasitophorous vacuole. The flagella were distributed within the main cytoplasmic mass and the condensed nuclei remained present at the periphery of the gamonts. This aberrant morphology, which was not observed with any other anticoccidial, again suggests abnormal differentiation in the course of microgametogenesis that apparently leads to the death of the microgamonts. This suggests that fertilization of macrogamonts may largely be precluded, thereby leading to the absence of oocyst formation and possibly also to degeneration of the unfertilized macrogamonts.

This study shows that single-dose diclazuril treatment (5 mg/kg) is completely effective against the asexual and sexual stages of *E. tenella* in chickens, indicating the lethal effects of this compound. This anticoccidial does not influence the growth phase, including nuclear divisions in schizonts and microgamonts, but interrupts their normal differentiation. The precise mechanism of action is unknown. As has been described for an azauracil derivative (Ryley et al., 1974), it is possible that diclazuril may be utilized in nucleic acid synthesis, allowing nuclear growth and division to take place in an apparently normal fashion, but inhibiting later phases of differentiation, thereby preventing further development.

#### ACKNOWLEDGMENTS

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## PULMONARY GRANULOMA FORMATION IN MURINE TOXOCARIASIS: TRANSFER OF GRANULOMATOUS HYPERSENSITIVITY USING BRONCHOALVEOLAR LAVAGE CELLS

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**ABSTRACT:** A major drawback to studying granuloma formation in murine toxocariasis is the ability of the second-stage larva of *Toxocara canis* to escape from a developing granuloma, migrate elsewhere, and initiate granuloma formation anew. In an attempt to circumvent this difficulty, 2 different *T. canis*-derived antigenic preparations were covalently attached to Sepharose 4B beads and embolized into the microvasculature of the lungs of CBA/J mice that had been infected 10 days previously with 25 *T. canis* ova. Both *T. canis* egg extract (TEE) and *T. canis* exoantigens (TEX) were able to elicit antigen-specific granulomas 6 days postembolization as determined by both histologic and morphologic criteria. Histologically, the eosinophil-rich granulomas forming around antigen-coated beads embolized into infected mice resembled the developing granuloma previously described forming around the second-stage larva. Attempts to transfer granulomatous reactivity in this model using either immune spleen cells or immune serum were unsuccessful. Successful transfer of granulomatous hypersensitivity was achieved using cells obtained by bronchoalveolar lavage of previously infected mice. The results suggest the feasibility of using this embolic model of granuloma formation in murine toxocariasis.

*Toxocara canis*, the cosmopolitan canine roundworm, is the etiological agent of human visceral larva migrans (VLM) syndrome. In the original identification of this organism as a human pathogen, Beaver et al. (1952) noted that chronic extreme eosinophilia was accompanied with granulomatous lesions in the livers of toddler-aged children in confirmed cases of VLM syndrome. Prior to the identification of *T. canis* there was a remarkable series of papers describing very young children with extremely high total leukocyte counts of which a substantial proportion were eosinophils associated with hepatic granulomata (Perlingiero and Gyorgi, 1947; Zeulzer and Apt, 1949; Mercer et al., 1950; Behrer, 1951). In all of these reports the authors had hinted that the pathology may have been due to helminthic infection but were unable to convincingly demonstrate any evidence of such. All, however, had described an eosinophil-rich granuloma with a constant histologic appearance.

Experimental studies on the development of the *T. canis* granuloma, especially in mice, have been performed by many laboratories (Burren, 1968; Kayes and Oaks, 1978; Parsons et al., 1986), and the finding of granulomas devoid of larvae or larval fragments is a common feature of all of these reports. Kayes and Oaks (1978) described an empty granuloma that was adjacent to another granuloma containing an apparently

viable second-stage larva (L<sub>2</sub>). Given the length of infection, they speculated that the larva had initiated granuloma formation at the first site and then escaped this site to become established in the latter site. Such observations also have been made by others. This ability of *T. canis* to escape encapsulation, migrate elsewhere, and initiate granuloma formation anew is an obstacle to the timed study of granuloma formation.

In an attempt to overcome this problem we have employed the pulmonary granuloma model of Carrick and Boros (1980), wherein CNBr-activated Sepharose 4B beads are covalently coated with either *T. canis* egg extract (TEE) or *T. canis* exoantigens (TEX, also known as excretory-secretory antigens) and embolized into the lungs of normal or *T. canis*-sensitized mice. We report here a comparison of the efficacy of the 2 antigen preparations to elicit antigen-specific pulmonary granulomatous inflammation and the successful transfer of granulomatous reactivity to uninfected syngeneic mice using bronchoalveolar lavage (BAL) cells but not spleen cells or hyperimmune serum derived from infected mice.

### MATERIALS AND METHODS

#### Mice

Female CBA/J mice were obtained from the Jackson Laboratories (Bar Harbor, Maine) and maintained in the vivarium facilities of the Department of Comparative Medicine, College of Medicine, University of South Alabama. These facilities conform to the N.I.H. guidelines for the use of laboratory animals. All mice were obtained as 12-15-g weanlings and were allowed to

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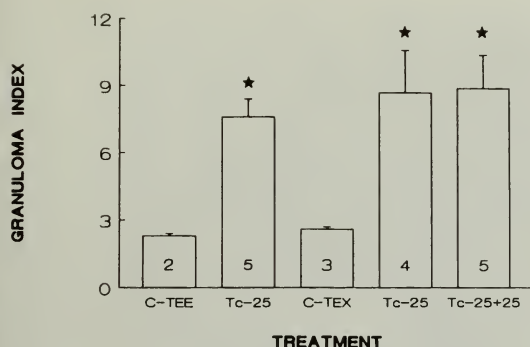


FIGURE 1. Pulmonary granuloma formation in CBA/J mice infected once with 25 *T. canis* ova (Tc-25) or infected twice at weekly intervals with 25 *T. canis* ova (Tc-25+25). Embolization of 7,000 TEE- or TEX-coated Sepharose beads was performed 10 days after the last infection, and lungs were removed for analysis 6 days later. The controls, designated C-TEE and C-TEX, represent the results of embolization of the designated bead type into uninfected, normal mice. Results are expressed as mean granuloma index (see Materials and Methods for explanation)  $\pm$  SEM. Numbers in base of bar indicate number of mice, and asterisks indicate statistical significance when compared with the appropriate control.

adapt to their quarters for at least 1 wk prior to inclusion in any experiments. Mice were allowed rodent chow and water *ad libitum*.

### Parasite

Female *T. canis* were obtained from the intestines of euthanatized stray puppies. The procedures for their culture and introduction into the mouse host have been described in detail previously (Kayes, 1984; Kayes et al., 1985). Briefly, eggs were cultured in 0.1 N  $H_2SO_4$  for 28 days in the dark at room temperature to allow for embryonation. Following microscopic verification that eggs contained infective second-stage larvae, the cultures were stored at 4 C until needed. Mice, under light ether anesthesia, were infected by gastric intubation with the specified number of ova.

### Antigens

The preparation of TEE was as described by Cypress et al. (1977). This preparation is an extract of homogenized, embryonated ova in borate buffer, pH 8.3. The TEX was prepared as described by de Savigny (1975) and was a gift of Dr. Robert Grieve, Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison.

### Transfer of immune cells or immune serum

Aseptic spleen cell suspensions were prepared and  $5 \times 10^7$  cells in 0.5 ml of RPMI-1640 were injected into uninfected recipient mice via the lateral tail vein. Hyperimmune sera were collected from mice 14 days after the last of 3 weekly infections with 100 *T. canis* ova (300 ova, total per mouse) and pooled. The specific activity of pooled normal mouse serum and the hyperimmune serum were determined as previously described (Kayes et al., 1985), using an IgG and IgM

TEX-specific ELISA assay. Pooled sera were assayed at a dilution of 1:100 and had  $A_{405/490}$  ratios of 0.076 and 0.421, respectively.

### Preparation of antigen-coated beads

Freeze-dried CNBr-activated Sepharose 4B beads (0.5 g; Sigma Chemical Co., St. Louis, Missouri) were hydrated for 15 min in 100 ml of 1 mM HCl, sized using 41- and 52- $\mu$ m Spectromesh macro filtration screens (Spectrum Medical Industries, Inc., Los Angeles, California) to achieve a uniform bead population of  $46 \pm 8 \mu$ m, and concentrated by centrifugation at 1,000 g, 4 C for 10 min. The supernatant was removed by aspiration and the bead pellet resuspended in 1 ml of the appropriate protein solution. TEX was used at a concentration of 1 mg/ml, pH 8.3, and TEE at 0.63 mg/ml, pH 8.3. The protein gel suspension was mixed overnight at 4 C on an end-over-end mixer. The gel suspension was again centrifuged and the pellet resuspended in 3 ml of 1 M ethanolamine (Sigma), pH 8.0, at room temperature for 2 hr on the same mixer to block unbound sites on the beads. The gel suspension was washed several times in sterile phosphate-buffered saline, pH 7.6, and then stored at 4 C until needed.

### Bronchoalveolar lavage

This procedure has been described in detail elsewhere (Kayes et al., 1987). The mice to be lavaged were exsanguinated under ether vapor, the thoracic cavity opened, and the trachea exposed by resecting the stylohyoid and omohyoid muscles. A silk ligature was secured around the upper trachea (to prevent reflux of the lavage). The lower trachea between the ligature and the lungs were entered with a 25-gauge, 0.5-in. (1.27-cm) needle attached to a small syringe containing  $Ca^{2+}$ ,  $Mg^{2+}$ -free phosphate-buffered (Dulbecco) saline (GIBCO, Long Island, New York) with 60 U of heparin/ml (DBSSH). The lungs were infused with 0.5 ml of DBSSH, and the infusate was aspirated back and deposited into a sterile ice-cold culture tube. The procedure was repeated until approximately 5 ml of lavage fluid was obtained. The cells were washed once in DBSSH and resuspended in the same for total cell and differential counting.

### Adoptive transfer of bronchoalveolar cells

Groups of cell donor mice were infected with 250 *T. canis* ova, and 10 days later they were exsanguinated and their lungs subjected to bronchoalveolar lavage. Syngeneic *T. canis*-naive mice were anesthetized with barbiturates and their tracheas surgically exposed. Using an insulin syringe with a 27-gauge needle, 100  $\mu$ l of sterile DBSS containing  $5 \times 10^6$  cells were injected via the trachea into the lungs. The incisions were closed with 9-mm wound clips and 4 hr after the mice revived from the anesthetic, 7,000 beads coated with either ethanolamine (control) or TEX were injected intravenously. In a series of 2 experiments, these mice were necropsied from 3 to 7 days after bead embolization.

### Histological procedures and granuloma measurements

Necropsy of mice bearing bead granulomas was accomplished 6 days postembolization after exsanguination under ether vapor. The chest cavity was opened



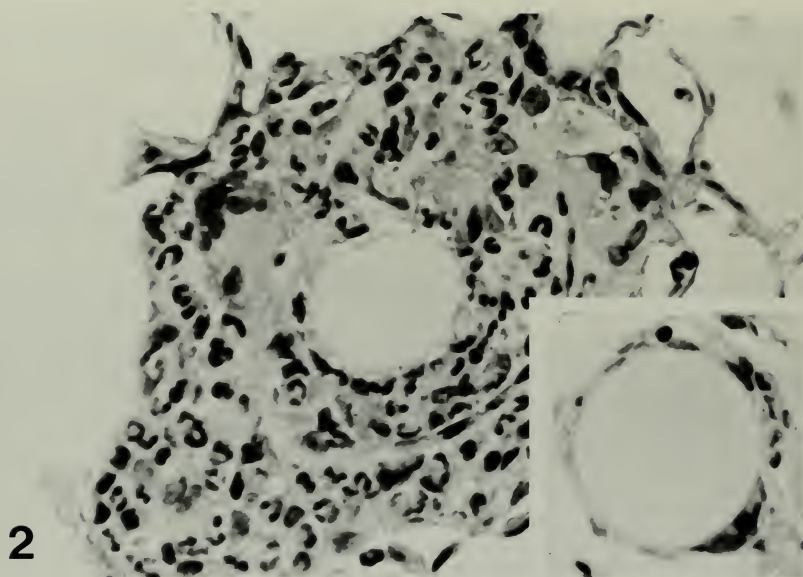


FIGURE 2. Photomicrograph of lung of CBA/J mouse infected with 25 *T. canis* ova and challenged 10 days later i.v. with TEE-coated Sepharose beads resulting in pulmonary embolization of the antigen-coated beads. Note the eosinophil-rich nature of the granuloma and the multinucleate giant cell (H&E,  $\times 550$ ). The inset depicts the granulomatous reaction around a TEE-coated bead in an uninfected control mouse (H&E,  $\times 550$ ).

and the lungs and trachea exposed. The lungs were perfused intratracheally with 1–2 ml of 10% buffered formalin. The lungs were then removed and further fixed by immersion. The tissues were washed, dehydrated through graded alcohols to 95%, embedded in glycol methacrylate (JB-4; Polysciences, Inc., Waring, Pennsylvania), and sectioned at  $2\ \mu\text{m}$ . All sections were stained with hematoxylin and eosin. Granuloma areas were measured using a video-based image analysis system (Bioquant; R&M Biometrics, Nashville, Tennessee) utilizing a vendor-supplied computer program, "Digitizing Morphometry." The granuloma index (G.I.) was determined by dividing the cross-sectional area of the granuloma by the cross-sectional area of the nidal bead for each granuloma so as to correct for how far off center the actual plane of sectioning occurred. Twenty-five granulomas per animal were measured and only those reactions containing a bead having a diameter of  $36\ \mu\text{m}$  or larger were included in the final calculations.

#### Statistical analysis

Morphometric data were analyzed by Student's *t*-test (serum transfer experiments) or single factor analysis of variance with multiple paired comparisons by Dunnett's method as the post hoc test. Significance was assessed in all cases at the  $P < 0.05$  level of confidence.

### RESULTS

#### TEE-elicited granulomatous inflammation in mice infected with 25 ova of *T. canis*

Uninfected control mice and mice infected with 25 embryonated ova 10 days previously received intravenous injections (i.v.) of 7,000 TEE-coated

beads in 0.5 ml of sterile borate-buffered saline. Six days after embolization the lungs were removed for morphometry. The results of 1 such experiment showing the ability of TEE-coated beads to elicit granulomatous inflammation are shown in the left half of Figure 1. TEE-coated beads embolized into the lungs of uninfected control mice elicited foreign body reactions consisting of 1–2 layers of macrophages and fibroblasts. The granuloma index was slightly greater than 2.0. In contrast, when TEE-coated beads were embolized into the lungs of infected mice there was over a 3-fold increase in the granuloma index. The histological appearance of this lesion was reminiscent of the developing *T. canis* lesion (Kayes and Oaks, 1978) in that it consisted of between 60 and 80% eosinophils with the remaining cells being macrophages, multinucleate giant cells (MNGC), lymphocytes, and occasionally, polymorphonuclear leukocytes (PMN's) (Fig. 2). Thus, TEE-coated beads elicit a *T. canis*-specific granulomatous response in *T. canis*-infected mice but not in uninfected control mice.

#### TEX-elicited granulomatous inflammation in mice infected with 25 ova of *T. canis*

Uninfected mice and mice infected with 25 *T. canis* ova once or twice at weekly intervals (50 eggs total) received i.v. injections of TEX-coated

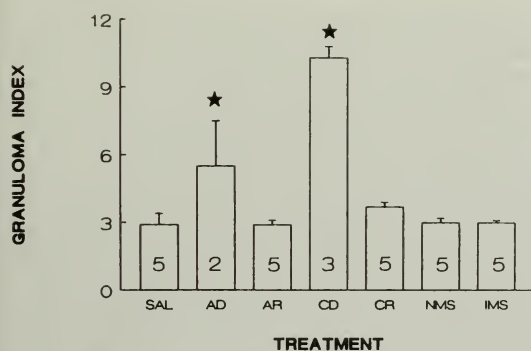


FIGURE 3. Transfer of immune spleen cells from donor mice infected with *T. canis* either 14 days acutely (AD), or 28 days chronically (CD), or normal mouse serum or immune mouse serum transferred into *T. canis*-naïve syngeneic recipients. The recipient groups are sham transfer recipients receiving saline (SAL) or recipients of the acute cells (AR) or chronic cells (CR) or normal mouse serum (NMS) or immune mouse serum (IMS) from mice multiply-infected with *T. canis*. Twenty-four hours after transfer, all mice received an embolizing i.v. injection of 7,000 TEX-coated beads, and the granuloma indices were determined 6 days later. Results are expressed as mean granuloma index  $\pm$  SE. The numbers in the base of the bars indicate the number of mice, and asterisks indicate statistical significance relative to the SAL control group.

beads 10 days after the last infective inoculum. After 6 days the lungs were removed for morphometric analysis. A representative experiment can be seen in the right half of Figure 1. TEX-coated beads embolized into uninfected mice resulted in a mean G.I. of 2.5. Both once-infected and twice-infected mice had mean G.I.'s of just under 9.0. There were no significant differences in the size or histological constituency of the TEX-bead granulomas that could be attributed to single or double infections. TEX-bead lesions resembled TEE-bead lesions as depicted in Figure 1, and consisted mostly of eosinophils, with the remaining cells being macrophages, MNGC's, lymphocytes, and PMN's.

#### Failure to transfer granulomatous reactivity using spleen cells from syngeneic mice infected for 14 or 28 days with *T. canis*

Groups of mice were infected with 100 *T. canis* ova and 14 or 28 days later, sterile single cell suspensions of their spleens were injected i.v. ( $5 \times 10^7$  cells/recipient) into syngeneic naïve recipients. Twenty-four hours later all recipients and infected donors, not previously sacrificed, received 7,000 TEX-coated beads i.v., and 6 days later their lungs were removed for morphometry. The results of 1 representative experiment (Fig.

3) show that regardless of whether mice received 14-day immune spleen cells, 28-day immune spleen cells, or a sham injection of sterile saline, the G.I. at 6 days postembolization was  $<3.0$ . Both acute and chronic donor animals had significantly elevated granuloma indices.

#### Failure to transfer antitoxocara granulomatous reactivity with immune mouse sera

One group of uninfected recipient mice received i.p. injections of 0.5 ml of normal mouse sera (NMS) and another group received the same volume of immune mouse sera (IMS). TEX-coated beads were embolized into the lungs 24 hr later, and after 6 days the lungs were removed for morphometry. The results of 1 of 2 experiments are shown as the last 2 bars of Figure 3. As can be seen, the IMS was no more effective at conferring granulomatous reactivity than was NMS. Six days after the i.p. passive transfer of 0.5 ml of normal mouse serum or hyperimmune serum, pooled serum collected from the recipients had  $A_{405/490}$  ratios of 0.081 and 0.193, respectively.

#### Transfer of granulomatous reactivity with bronchoalveolar lavage cells from syngeneic mice infected for 10 days with *T. canis*

The results, shown in Table I, indicate that cells obtained directly from *T. canis*-inflamed lungs by bronchoalveolar lavage can adoptively confer granulomatous reactivity to previously unexposed mice. The lesions around TEX-coated beads were significantly larger than control beads as early as 3 days postembolization and reached their peak size after 6 days. Histologi-

TABLE I. Endotracheal adoptive transfer of *Toxocara canis*-specific granulomatous hypersensitivity to syngeneic normal mice using bronchoalveolar lavage cells.

Bead coated with:		Mean $\pm$ SE	
		Granuloma index	Granuloma area ( $\mu\text{m}^2$ )
Experiment I			
Ethanolamine	(2)*	2.4† $\pm$ 0.24	4,122 $\pm$ 244
TEX‡—6 days	(2)	5.3 $\pm$ 0.64§	8,533 $\pm$ 407§
Experiment II			
Ethanolamine	(4)	2.6 $\pm$ 0.04	3,788 $\pm$ 176
TEX—3 days	(5)	3.8 $\pm$ 0.29§	5,879 $\pm$ 488§
TEX—5 days	(4)	4.1 $\pm$ 0.05§	5,955 $\pm$ 662§
TEX—7 days	(3)	4.1 $\pm$ 0.28§	7,278 $\pm$ 467§

\* Number of mice per determination.

$\dagger$  Granuloma index = area of granuloma/area of coated bead.

$\ddagger$  *Toxocara* exoantigens.

§  $P < 0.05$ ; 1-way ANOVA (TEX vs. ethanolamine).



cally, the granulomata forming around TEX-coated beads contained approximately 50% eosinophils with the remaining cells being epithelioid macrophages, lymphocytes, and some neutrophils.

### DISCUSSION

Of the 2 antigen preparations studied, TEX has been more extensively characterized. Neutral proteases have been reported in mechanically hatched larvae but not in preparations of TEX (Badley et al., 1987a), and therefore, these proteases would be expected to be found in TEE preparations used in the present studies as well. TEX has been analyzed by several laboratories (Sugane and Oshima, 1983; Maizels et al., 1984, 1987; Meghji and Maizels, 1986; Badley et al., 1987a) and, depending on what preparative methodologies were employed, contained from 4 to 6 to more than 20 bands on SDS-polyacrylamide gel electrophoresis. Interestingly, Sugane and Oshima (1983) isolated a glycoprotein from TEX with a molecular weight of 35 kDa that was both immunogenic and allergenic. Denaturation of this molecule destroyed the allergen but not the immunogen. Subsequent studies of this protein indicated that it could elicit peripheral blood eosinophilia and activate complement when administered by osmotic pump implanted in the peritoneal cavity (Sugane and Oshima, 1984). Maizels et al. (1984) have shown that some of the glycoproteins found in TEX can also be detected on the surface of *T. canis* larvae. Thus, there is good reason to consider the use of TEX preparations for *in vivo* studies of granuloma formation in experimental toxocariasis.

Rabbits immunized with TEX produced antibodies that recognized patterns of TEX bands on nitrocellulose (western blots) that were very similar to those recognized by sera from natural or experimental infections (Badley et al., 1987b). *Toxocara canis*-immune serum has been shown to mediate the adherence of eosinophils to the surface of L<sub>2</sub> *T. canis* larvae. However, absorption of this serum by TEX completely abrogated the ability of this immune serum to mediate eosinophil adherence (Badley et al., 1987b). TEX also has the peculiar ability to remove anti-human blood group A and B antibodies from typing antisera (Smith et al., 1983), and this is consistent with the long known observation that patients infected with *T. canis* often have elevated anti-A and anti-B isoagglutinins.

The results presented in this study show the

feasibility of using the pulmonary granuloma paradigm of Carrick and Boros (1980) to study granuloma formation in murine toxocariasis. In addition, the results indicate that both of the antigenic preparations, TEE and TEX, are equally granulomagenic as determined by the measurement of mean cross-sectional area of the elicited lesions and more importantly, the histological appearance of the lesion, itself. However, it should be mentioned that this bead granuloma only resembles the initial granuloma forming around migrating L<sub>2</sub> in that well over half of the participating cells are eosinophils (Kayes and Oaks, 1978). However, unlike the living worm, which continues to elaborate antigenic materials, the bead would not be expected to sustain the granuloma once its bound antigens are enzymatically degraded.

Previously, it has been shown that infection of CBA/J mice with just 25 *T. canis* eggs results in a 6-fold increase in the number of circulating eosinophils. Such an infection also leads to a doubling of the spleen size, a 4–5-fold augmentation of Con A-elicited splenic lymphocyte transformation, and a significant TEE-elicited lymphocyte transformation. This infection also elicited a significant circulating antitoxocaral antibody at 14 days postinfection as compared to uninfected, age-matched control mice (Kayes et al., 1985). More recently, it has been demonstrated (Kayes, 1986) that when beads coated with methylated bovine serum albumin (MBSA) are embolized into the lungs of CBA/J mice infected with 250 *T. canis* ova, large, florid, eosinophil-rich granulomas develop that resemble the lesions described in this study. However, when MBSA-coated beads were embolized into the lungs of mice infected with only 10 *T. canis* ova, foreign body granulomas developed that contained no eosinophils (Kayes, 1986). The former reaction taking place in mice infected with 250 ova was interpreted as a nonspecific stereotypic response occurring in a hyperreactive lung. The latter result confirms the antigen-specific nature of TEE- and TEX-bead granulomas. Beads coated with *T. canis*-derived antigens elicited eosinophil-rich lesions only in infected mice and not in control mice. The substitution of the nonparasite-derived protein, MBSA, for *T. canis* antigens to coat the beads elicited nothing more than foreign body granulomata in mice infected with 10 eggs. These last 2 observations suggest that not just any protein will elicit eosinophilic granuloma in this paradigm.

The inability to transfer granulomatous inflammation with either immune cells or serum in CBA/J mice is disconcerting. Concurrent immunological studies performed on both the donor and recipient mice indicated that donor mice were, in fact, immune to *T. canis*, whereas the recipient mice were indistinguishable from uninfected controls (data not shown). The most likely explanation for this is that the transfused lymphocytes contained a substantial population of cells that were nonspecifically activated and hence contained too few *T. canis*-specific cells to be detectable in the bead granuloma assay or the lymphocyte transformation assay. Evidence for this derives from 2 sources. First, we have previously shown by several different criteria that CBA/J mice infected with 25 or more *T. canis* ova are nonspecifically hyperreactive (Kayes, 1986). Second, we have been unable to consistently demonstrate very good TEX-elicited lymphocyte transformation responses using spleen cells from infected mice, which may suggest that there are too few antigen-specific lymphocytes recognizing TEX. This has been demonstrated recently by showing that the ratio of the TEX-stimulated lymphocyte blastogenesis reaction to the Con A-stimulated lymphocyte blastogenic reaction was <5% when spleen cells from *T. canis*-infected mice were tested. However, when lymphocytes recovered from the lungs of *T. canis*-infected mice by bronchoalveolar lavage were evaluated in the same manner and exposed to TEX in the lymphocyte transformation assay, there were strong and consistent responses and the TEX: Con A ratio was 4–19-fold greater than for spleen cells (Kayes et al., 1987). This suggests that at least in the CBA/J strain there is an accumulation of antigen-reactive T cells in the periphery where the antiparasitic responses are occurring and that the spleen may not be a good source of such lymphocytes. The possibility also must be considered that both immune cells and serum are needed together. Because both immune cells alone or serum alone were not the least bit different from uninfected controls, we believe that this is not a likely possibility and this experiment was not attempted. Studies in different inbred strains of mice suggest hyperreactivity may not be a common feature of murine toxocariasis (Jones and Kayes, unpubl. obs.), and this may facilitate the adoptive transfer of *T. canis*-focused granulomatous reactivity in other strains using spleen cells.

In conclusion, we have shown that Sepharose

4B beads, covalently coated with *T. canis*-derived proteins and embolized into the pulmonary microvasculature of mice previously exposed to *T. canis*, develop antigen-specific granulomas. This granulomatous reactivity can be adoptively transferred to syngeneic *T. canis*-naïve mice using cells obtained by bronchoalveolar lavage but not by spleen cells or immune serum. This model should permit detailed studies of the immunoregulation and chemical mediation of this cell-mediated delayed hypersensitivity lesion.

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## CHANGES IN ALBUMIN LEVELS IN BLOOD AND URINE OF *MICROTUS MONTANUS* CHRONICALLY INFECTED WITH *TRYPANOSOMA BRUCEI GAMBIENSE*

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**ABSTRACT:** Serum albumin and glucose concentrations and urinary excretion of alpha-keto acids and proteins were determined in samples obtained throughout a chronic *Trypanosoma brucei gambiense* infection in *Microtus montanus*. An increase in urinary excretion of alpha-keto acids and proteins during the terminal stage of disease was accompanied by a decrease in serum glucose concentration. This terminal hypoglycemia reflected a depletion of liver glycogen in most animals. In contrast (and the major focus of this study) serum albumin concentration was decreased by the second week of infection and in the final sample obtained was less than 50% of that measured in preinfection samples. Female animals survived approximately 1 wk longer than males and were less susceptible during the acute phase of disease. This relative resistance was most likely due to the fact that female animals were relatively more efficient in limiting parasitemia during the first week of infection. The similarity between humans and voles in terms of protein and alpha-keto acid excretion and changes in serum concentrations of glucose and albumin during trypanosome infection further validate the use of *Microtus* as an experimental model for trypanosomiasis in humans.

Establishing the validity of experimental animals as accurate models for African trypanosomiasis in humans requires considerable information concerning the clinical and pathological changes that occur during infection. Animal models that exhibit changes similar to those occurring in man will ultimately serve in experiments designed to unravel mechanisms of human pathogenesis. Although the primary mechanism of pathogenesis in African trypanosomiasis is thought to result from immune dysfunction (Shapiro and Pearson, 1986), a number of physiological changes, including an alteration in aromatic amino acid metabolism, have been implicated as well (Seed et al., 1983).

The mountain vole, *Microtus montanus*, was established as a model for trypanosomiasis in humans (Seed and Hall, 1980). As in man, these animals excrete high urinary concentrations of alpha-keto acids, suggesting a common defect in metabolism (Seed et al., 1982; Hall et al., 1985). It has recently been reported that trypanosome-infected voles exhibit terminal hypoglycemia that is accompanied by depletion of liver glycogen (Seed et al., 1987). In a continuing effort to characterize trypanosomiasis in *Microtus* and in order to provide insight into possible mechanisms

of pathogenesis, we have examined serum samples obtained throughout infection for changes in glucose and albumin levels and urine samples for the presence of albumin and total proteins. The changes in albumin and protein levels in urine were correlated with changes in blood glucose and urine alpha-keto acid levels.

### MATERIALS AND METHODS

The history and biological characteristics of the TxTat 1.0 clone of *T. b. gambiense* used in these experiments have been described previously (Seed et al., 1977). Cells obtained from an irradiated CD-1 mouse (800 rad, <sup>137</sup>Cs source) were purified by anion exchange chromatography (Lanham and Godfrey, 1970) and maintained as a frozen stablate. All experimental infections were established by injecting mountain voles (8-14 wk of age) intraperitoneally with  $5 \times 10^3$  cells from this stablate. Details concerning the care and maintenance of voles have been described previously (Seed and Hall, 1980).

Parasitemias were estimated from Giemsa-stained blood smears through the use of a standard curve that correlates the number of cells in a blood smear with the number of cells per ml of whole blood (Seed et al., 1987). Samples were obtained from tail blood collected every fourth day beginning 4 days after infection. In order to obtain serum samples, blood was collected from the retro-orbital sinus into heparin-coated pipets, microfuged for 20 sec (10,000 g), and the serum removed for storage at -70 C. Serum samples were collected every fourth day between 4 and 6 P.M. beginning 4 days prior to infection. Serum glucose concentrations were determined spectrophotometrically as described by Raabo and Terkildsen (1960) using a standard kit (Sigma).

Urine was collected from individual animals placed in metabolic chambers (Fisher) for 9 hr from 11 P.M.

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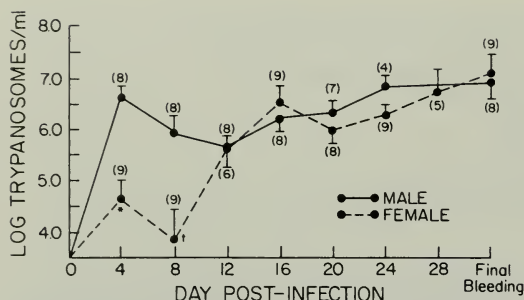


FIGURE 1. Average parasitemia in male (solid line) and female (broken line) *Microtus montanus* infected with *T. b. gambiense*. Data are from animals that developed chronic infection (survived >14 days). Numbers in parentheses equal number of animals examined. Results are expressed as average  $\pm$  SD. Significance was  $P \leq 0.001$  (\*) and  $P \leq 0.05$  (†).

to 8 A.M. Following 5 consecutive nights in which animals were acclimated to the chambers, urine was collected from animals every second night. Animals had access to food and water during the collection period. Urine was collected in 2.0 ml of 0.8% boric acid, centrifuged at 1,000 g to remove particulate matter, and the supernatant stored at  $-70^{\circ}\text{C}$ . Total urinary protein concentrations were determined with the Coomassie protein assay (BioRad) using IgG as the standard (Bradford, 1976).

In order to quantitate the albumin concentration in serum and urine, albumin was purified from whole vole sera by affinity chromatography using Blue Sepharose CL-6B (Pharmacia). Albumin was eluted with 1.5 M NaCl in 0.2 M phosphate, pH 7.1 (Travis et al., 1976). Using rabbit antisera to whole vole serum, eluted material was revealed as a single precipitin arc by immunoelectrophoretic analysis. In addition, a single band with an apparent molecular weight of 66 kDa was observed in Coomassie-stained polyacrylamide gels. Antisera to purified vole albumin was developed in a New Zealand white rabbit injected with 2.0 mg protein emulsified in complete Freund's adjuvant. Following 2 further injections of 2.0 mg protein at 3-wk intervals using Freund's incomplete adjuvant, the animal was sacrificed and serum collected. Albumin concentration in urine and serum was determined by radial immunodiffusion in agarose gels (Crowle, 1973). For each gel, 2-fold dilutions of purified vole albumin were included in order to construct standard curves. The limit of detection of this assay was 0.15 mg albumin/ml. The concentration of purified albumin was determined with the Coomassie protein assay using bovine serum albumin as the standard. The concentration of individual alpha-keto acids in urine was measured by HPLC. Details of the extraction and assay protocols are provided elsewhere (Seed et al., 1982).

Urinary excretion of protein was monitored with the Coomassie protein assay. The concentration of total alpha-keto acids in freshly collected urine was determined with the dinitrophenylhydrazine assay (Neuberg et al., 1952). Animals were sacrificed when their urinary excretion of both protein and alpha-keto acids was elevated to high levels ( $>3.5$  mg protein/9 hr,  $>50$

$\mu\text{mol}$  total alpha-keto acids/9 hr). Immediately prior to sacrifice a final serum sample was collected. At sacrifice the major organs were removed, weighed, and fixed for pathological analysis. The results of this analysis will be the subject of a subsequent report. A portion of the liver was snap frozen in liquid nitrogen for analysis of glycogen concentration using the method of Roehrig and Allred (1974). The final serum collection and the processing of organs occurred between 1 and 3 P.M.

Data were analyzed for statistical significance using the Wilcoxon's 2-sample and signed-ranks tests.

## RESULTS

A number of differences in the response of individual mountain voles to infection with *T. b. gambiense* were apparent. Although certain of these differences were perhaps due to the fact that the vole colony is maintained as an outbred population, host sex also appeared to influence the infection. Therefore, the data are, when available, presented separately for male and female animals.

Animals that survived less than 14 days of infection (38% or 5 of 13 males; 10% or 1 of 10 females) did not exhibit an increase in excretion of protein or a decrease in serum glucose levels. Of the 6 animals included in this group, only a single male survived greater than 9 days and this animal exhibited a decrease in serum albumin concentration of 40% on day 12 of infection. Serum albumin levels in the other acutely infected animals were not decreased. In contrast, the concentration of the 4 alpha-keto acids was elevated in samples collected within the 24-hr period preceding the death of those animals with acute infection. In addition, the parasitemia determined from blood smears obtained 24 hr preceding death in those animals that survived less than 14 days of infection indicated that these animals failed to control the initial wave of parasites.

Although there were only minor differences in the parasitemias of male and female animals 12 or more days postinfection, the number of trypanosomes present in the blood of female animals was significantly less than in male voles 4 ( $P \leq 0.001$ ) and 8 ( $P \leq 0.05$ ) days following infection (Fig. 1). For those animals that developed chronic infections (>14 days), female animals survived significantly longer than males ( $30.7 \pm 6.0$  vs.  $23.4 \pm 3.2$ ,  $P \leq 0.005$ ).

The serum glucose concentrations for the chronically infected male animals did not decrease significantly from preinfection levels until

TABLE I. Terminal serum glucose and liver glycogen concentrations in *Microtus montanus* chronically infected with *T. b. gambiense*.

	$\bar{x} \pm SD$			
	n	Males	n	Females
Serum glucose (mg/dl)				
Infected	8	57.35 $\pm$ 20.91*	—	—
Control	7	84.33 $\pm$ 12.99	—	—
Liver glycogen ( $\mu$ g/mg liver)				
Infected	7	11.84 $\pm$ 5.08†	8	24.14 $\pm$ 17.17
Control	3	46.74 $\pm$ 2.41	2	43.75 $\pm$ 0.47

\*  $P \leq 0.02$  for glucose levels in serum from infected vs. control animals. Samples from infected animals collected immediately prior to sacrifice. No statistical difference for samples collected prior to final sample.

†  $P \leq 0.05$  for glycogen levels in livers from infected vs. control male animals. Note that although not statistically significant, 5 out of the 8 female animals had greatly reduced glycogen levels ( $\bar{x} = 12.31$ ,  $SD = 7.05$ ).

the final sample taken immediately prior to sacrifice ( $P \leq 0.02$ ) (Table I). This decrease in glucose concentration was correlated with a significant decrease in liver glycogen levels in male animals ( $P \leq 0.05$ ) (Table I). Although a majority of female animals also exhibited a decrease in glycogen level, the livers of 3 of 8 animals contained relatively normal glycogen concentration.

Serum albumin concentration was significantly decreased from preinfection levels by day 12 of infection in chronically infected animals ( $P \leq 0.005$ ). By day 20 the average albumin concentration had fallen to below 20 mg/ml (Fig. 2). Although the average serum albumin concentration in male animals was greater both prior to and during infection than in female animals, this

difference was statistically significant only for samples obtained 8 days postinfection and at sacrifice ( $P \leq 0.005$  and  $P \leq 0.05$ , respectively). For 6 of 17 animals, albumin was not detected in urine samples collected during the infection. For the remaining animals albumin was not detected in urine earlier than 24 days of infection. In the final urine collection the amount of albumin excreted for the 11 animals was  $7.4 \pm 5.8$  mg/9 hr. Albumin accounted for only about 30% of the total protein present in these samples.

Protein levels in urine of chronically infected animals were significantly increased from preinfection levels by day 16 of infection ( $P \leq 0.005$ ) (Fig. 2). Female animals excreted less total protein than males prior to and throughout the infection ( $P \leq 0.025$  for each sampling).

Pyruvate and alpha-ketoglutarate levels in urine of chronically infected animals were also significantly elevated by day 20 postinfection ( $P \leq 0.05$ ). HPLC analysis of urine samples from male animals confirmed the presence of relatively high urinary concentrations of alpha-ketoglutarate, pyruvate, phenylpyruvic acid, and 4-hydroxyphenylpyruvic acid during the latter stage of infection (Table II). The urines from all chronically infected animals that were monitored with the dinitrophenylhydrazine and Coomassie assays contained elevated concentrations of total alpha-keto acids and proteins, respectively, during the latter stages of infection. Serum albumin and glucose concentrations and urinary excretion of total alpha-keto acids and proteins were not altered in samples collected from uninfected animals monitored for a 4-wk period.

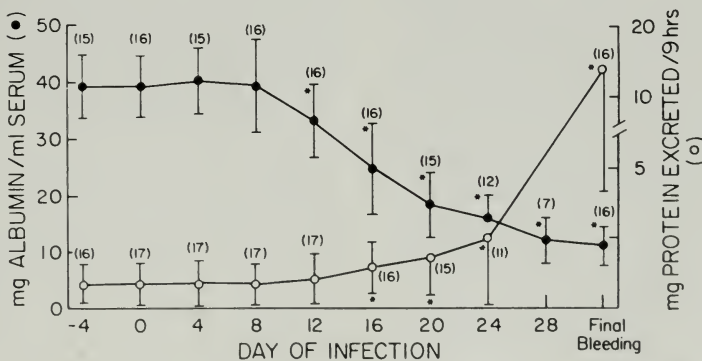


FIGURE 2. Serum albumin concentration (closed circles) and protein levels in urine (open circles) of *Microtus montanus* chronically infected with *T. b. gambiense*. Numbers in parentheses indicate the number of samples examined. Data are compiled from samples obtained from male and female animals. Results are expressed as average  $\pm$  SD. Significance was  $P \leq 0.005$  (\*).



TABLE II. Average alpha-keto acid excretion by chronically infected male *Microtus montanus*.

Day of infection	n	Alpha-keto acids ( $\mu\text{mol}$ excreted/9 hr)*			
		Alpha-ketoglutarate	Pyruvate	Phenylpyruvate	p-hydroxyphenylpyruvate
-4	7	0.24	2.04	N.D.†	N.D.
0	5	0.34	1.91	N.D.	N.D.
4	8	0.30	2.07	N.D.	N.D.
16	7	0.38	1.90	N.D.	N.D.
20	5	1.60‡	6.28‡	N.D.	N.D.
Final	6	51.59§	21.94§	21.96§	39.08§

\* Values determined by assay of urine by HPLC as described in Materials and Methods.

† Not detected. Limit of detection for individual keto acids is 0.1  $\mu\text{mol}$ .

‡  $P \leq 0.05$ .

§  $P \leq 0.025$ .

## DISCUSSION

The infection of *M. montanus* with monomorphic clones of African trypanosomes differs from that observed in murine models in that the disease in voles is usually chronic, is accompanied by behavioral changes, and is characterized by relatively low parasitemia (Seed and Hall, 1980). In these respects the disease in voles is similar to that in humans. In view of these similarities, the purpose of the present study was to more fully examine the course of trypanosomiasis in voles in an attempt to further establish this animal as a model for human trypanosomiasis.

Approximately 1 of 4 infected animals did not survive the initial 2 wk of infection and the majority of these acute infections were of less than 6 days duration. In those animals that developed chronic infection there was an increase in protein excretion beginning during the third week of infection that was accompanied by an increase in excretion of alpha-keto acids. The increase in urinary excretion of the 4 alpha-keto acids in addition to an increase in excretion of a number of aromatic amino acid catabolites has been reported previously (Seed et al., 1982; Hall and Seed, 1984). Although high urinary protein concentration indicates kidney dysfunction, perhaps resulting from immune complex-mediated glomerulonephritis as occurs in other animal models (Facer et al., 1978; Lindsley et al., 1980; Van Marck et al., 1981), it does not appear that excretion of alpha-keto acids is due to kidney damage. The urines from uninfected animals injected intraperitoneally with a mixture of the 4 alpha-keto acids were strongly positive using the dinutrophenylhydrazine assay, but were negative for

protein within 24 hr of injection. Thus, it appears that the mechanism that underlies the excretion of keto acids and the mechanism that results in kidney dysfunction may be entirely separate. Organic aciduria is also characteristic of African trypanosomiasis in humans as is proteinuria (Seed et al., 1983).

Terminal hypoglycemia in trypanosomiasis is known to result from an alteration in host energy metabolism and to a certain extent reflects the high energy requirement of trypanosomes (Mercado and Von Brand, 1960; Marciacq and Seed, 1970). The results from this study indicate that glucose levels are maintained at normal levels until the terminal stage of infection. Hypoglycemia is observed during the terminal stage of a number of infections (Wolfe, 1981) and the pattern in trypanosome infected voles is consistent with that reported in studies of *T. congolense*-infected cattle (Welde et al., 1974) and *T. brucei*-infected rabbits (Goodwin and Guy, 1973). It is possible that the terminal hypoglycemia results from the excretion of high concentrations of potential energy sources, particularly pyruvate and alpha-ketoglutarate. This loss would serve to emphasize the competition between host and trypanosome for glucose. The relative decrease in liver glycogen levels in the majority of infected animals suggests that this particular energy source is heavily utilized. An increase in glycogen mobilization is in agreement with the findings of previous studies (Mercado and Von Brand, 1960; Marciacq and Seed, 1970; Ashman and Seed, 1973; Seed et al., 1987). At present it is not known how early in the infection glycogen levels are decreased and virtually nothing is known about carbohydrate metabolism in infected humans. However, as with most inflammatory infections, it is probable that hypoglycemia in trypanosome-infected voles correlates temporally to the depletion of liver glycogen (Wolfe, 1981).

The major focus of this study and the data new to our understanding of the physiological response of voles to trypanosomiasis is the decrease in serum albumin levels. A decrease in serum albumin concentration is also characteristic of an acute inflammatory reaction (Powanda and Moyer, 1981) and has been reported in studies of experimental animals with trypanosomiasis (Goodwin and Guy, 1973; Welde et al., 1974; Luckins, 1975; Valli et al., 1980). Serum albumin levels are reported to be normal (Lambert et al., 1981) or decreased (Gall, 1956) in infected humans. Although a decrease in serum albumin

concentration in infected voles was initially thought to reflect the decreased availability of tryptophan and tyrosine that occurs in these animals (Newport et al., 1977), an analysis of serum protein profiles by polyacrylamide gel electrophoresis suggests that albumin is the only major serum protein to be decreased in concentration during infection. Nevertheless, this decrease is probably significant in that the normal functions of albumin, including transport of fatty acids, a number of hormones, tryptophan, cysteine, and zinc, are, in effect, limited. As in a number of other infectious diseases, a decrease in albumin levels during trypanosomiasis is probably necessary to counterbalance a rise in serum specific gravity that results from an increase in immunoglobulin synthesis (Peters, 1975). Although a certain percentage of the decrease in albumin concentration in voles may have resulted from albumin collection in extravascular spaces, this decrease is more likely due to a reduction in albumin synthesis or possibly an increase in albumin catabolism. Terminal albuminuria accounts for a fraction of the observed decrease in serum albumin, but a greater than 50% reduction in serum albumin concentration occurs prior to the onset of proteinuria.

The lower parasitemia in female voles during the early stage of infection and their increased survival times compared to male animals suggests that host sex influences innate resistance to trypanosomiasis. Similar differences have been reported by Greenblatt and Rosentreich (1984) in mice infected with *T. b. rhodesiense*. Glycogen levels and urinary protein concentrations differed between male and female animals, but other factors, including hormonal influences, are likely to be involved as well. Sex-related differences in susceptibility to disease have been noted for a variety of infections including a number caused by other parasitic organisms (Hauschka, 1947; Frayha et al., 1971; Landolfo et al., 1981; Giannini, 1986).

Although establishing the validity of experimental hosts as models for trypanosomiasis is difficult owing to the lack of clinical data regarding African trypanosomiasis in humans, it would appear on the basis of the information available that the infection in voles is similar to that in humans in terms of parasitemia, protein and alpha-keto acid excretion, and serum albumin concentration. With the exception of organic aciduria, which is not characteristic of other infectious diseases, the physiological changes re-

ported are typical of an acute inflammatory type reaction. Excluding the decrease in albumin levels, major physiological changes are not observed until late during infection. In view of the importance of albumin as a transport protein, the significant decrease in serum albumin concentration undoubtedly signals a major physiological adaptation to disease.

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## **SCHISTOSOMA MANSONI: CHARACTERIZATION OF CLONES MAINTAINED BY THE MICROSURGICAL TRANSPLANTATION OF SPOROCCYSTS**

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**ABSTRACT:** Five male and 5 female clones of *Schistosoma mansoni* were established and maintained for 3 yr by the serial microsurgical transplantation of sporocysts from infected to uninfected *Biomphalaria glabrata* snails. The clones were initially derived from 10 randomly selected snails with monomiracidial infections. Clones were characterized by several criteria, including their infectivities for mice and snails, their cercarial outputs, and their ability to produce immunity in mice. The mean infectivities of individual clones in mice ranged from 26 to 44%, and were highly consistent within each clone. The infectivities of cloned sporocysts in snails ranged from 44 to 100% and were also highly consistent within clones. Mean cercarial outputs from individual clones ranged from 450 to 4,300 per snail. In mice, clones differed significantly from each other in their ability to immunize and in their susceptibility to immunity. Each clone was unique and did not appear to differ with time or subpassaging through snails, suggesting that the differences had a genetic basis.

The schistosomes have a complex life cycle in which sexually reproducing stages in the vertebrate definitive host alternate with asexually reproducing forms in the molluscan intermediate host. Under natural conditions intramolluscan parasites derived from a single miracidium are genetically unique, and all adult schistosomes derived from them are members of a single clone. However, the life span of any clone is limited to the longevity of its snail host. An infected snail releases varying numbers of cercariae on different days, so its contribution to the cercarial pool varies from day to day. Also, a snail is often infected by more than 1 miracidium, and each miracidium makes a variable relative contribution to the pool. Infected snails have a high mortality that usually results in a rapid turnover of snails and the cercariae derived from them. It follows that each experiment employs schistosomes that differ genetically, producing results that are difficult to compare between different experiments and laboratories.

Variation in such factors as fecundity, prepatent period, and pathogenicity have been examined in different geographical isolates of *Schistosoma mansoni* (Saoud, 1966; Warren, 1967; Anderson and Cheever, 1972; Powers and

Cheever, 1972; Kassim et al., 1979), but variation within the common laboratory strain (PR-1) has never been rigorously studied, although LoVerde et al. (1985) reported that it appears uniform by isoenzyme analysis. Such studies are essential for an adequate understanding of schistosome biology and immunology.

Two theories on schistosome immunology have emerged: (1) variability in schistosome immunity is due to heterogeneity in the host (Dean et al., 1981; Simpson et al., 1983; Yong et al., 1983); and (2) the variability is due to heterogeneity in the schistosomes (Smith and Clegg, 1979). It is most likely that genetic variability in both the host and parasite are of great importance (Colley and Freeman, 1983; Dean, 1983; Cohen and Eveland, 1984a).

Microsurgical transplantations of intramolluscan hermaphroditic trematodes (Dönges, 1963, 1968; Heyneman, 1966) and schistosomes (Chernin, 1966) have been described. It has also been demonstrated that daughter sporocysts of schistosomes can produce additional generations of daughter sporocysts (Theron and Jourdan, 1979; Jourdan et al., 1980; Nojima et al., 1980), and that sporocysts can be passaged repeatedly through snails (Jourdan and Theron, 1980; Cohen and Eveland, 1984a). With the development of a procedure for the long-term maintenance of clones of *S. mansoni* by the serial microsurgical transplantation of sporocysts from infected to naive *Biomphalaria glabrata* snails, it is possible to obtain unlimited numbers of cercariae of any clone and to extend the life span of any clone beyond the lifetime of the original monomira-

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cidially infected sporocyst donor (Jourdane et al., 1980; Cohen and Eveland, 1984a). The purpose of this study was to characterize randomly selected clones of *S. masoni* maintained by the microsurgical technique. Four fundamental traits of the schistosomes were examined for variability within and between clones; their infectivity (recovery rate) for mice, their infectivity for snails, their cercarial outputs, and their ability to immunize mice.

## MATERIALS AND METHODS

### Parasites and hosts

Puerto Rican-1 (PR-1) *Schistosoma masoni* used in the experiments was established in 1950 from 170 naturally infected *Biomphalaria glabrata* from Arecibo, Puerto Rico (Fletcher et al., 1981), and maintained routinely in our laboratory for approximately 10 yr in M line *Biomphalaria glabrata* as previously described (Cohen et al., 1980). Ten clones (males 1-5 and females 1-5) were established and maintained by serial microsurgical transplantations as reported by Cohen and Eveland (1984a). Six- to 8-wk-old outbred female CD-1 mice (Charles River, Wilmington, Massachusetts) were used.

**Cercarial collection and counting.** Cercariae were collected from infected snails that were routinely maintained in plastic trays at 26-28°C in the dark and fed *ad libitum* on washed romaine lettuce. For experiments, snails were exposed at ambient room temperature to fluorescent light for 1 hr following a 48-hr dark period. Cercariae were always used within 3.5 hr following their emergence from snails, because cercarial infectivity has been reported to decrease by 5 hr after emergence (Olivier, 1966; Lawson and Wilson, 1983). Cercarial suspensions were mixed with a magnetic stirrer, 5 samples (10-100 µl) were removed, and the cercariae were stained with Lugol's iodine and counted.

### Mouse infection

Mice were anesthetized with 0.01 ml/g body weight of a 10-mg/ml solution of sodium Nembutal (Rugby Laboratories, Rockville Center, New York) in 0.85% saline. For cercarial infectivity studies and immunization challenge infections cercarial suspensions were placed on coverslips, and the exact number of cercariae on each coverslip was determined. The coverslips were inverted, placed on the moistened, shaved mouse abdomens, and left in place for 45 min. Worms were collected 6 wk later by perfusing the livers of mice anesthetized with Nembutal containing heparin, then washed free of blood by sedimenting them repeatedly in cold 0.85% saline and counted. "Cercarial infectivity" is the recovery rate, i.e., the percent of cercariae that developed into adult worms.

### UV attenuation of cercariae

Methods were similar to those used by other investigators (Ghandour and Magid, 1978; Dean et al., 1983), who have shown that ultraviolet (UV) irradiation can attenuate cercariae and that UV-attenuated cercariae can immunize mice against challenge with

nonirradiated cercariae. An S-68 Mineralight short-wave UV lamp calibrated with a UVX Short Wave UV Meter (both from Ultra-Violet Products, San Gabriel, California) was used to irradiate cercariae suspended in 15 ml of SMB water (Cohen and Eveland, 1984a) in 9-cm glass petri dishes.

### Immunization of mice

Mice were put into plastic restrainers and their tails were immersed for 1.0 hr in a test tube containing 500-1,000 UV-attenuated cercariae (Eveland and Ritchie, 1971). Four weeks later the mice were divided into 2 groups of 5-7. The first group (IC = immunized-challenged mice) was challenged by percutaneous abdominal exposure to normal cercariae. The second group (I = immunization infectivity controls) was perfused 5-6 wk after immunization. At the time of challenge, normal mice (C = challenge infectivity controls) were simultaneously exposed to the same pool of cercariae as the IC group to determine the infectivity of the challenge cercariae. The IC and C groups were perfused 6 wk after challenge. Immunity was measured by the reduction in the number of challenge worms in the IC group as compared to nonimmunized controls, calculated by the following formula:

$$\% \text{ protection} = 1 - \frac{IC - I}{C} \times 100.$$

Because the cercariae were completely attenuated (see Fig. 5),  $I = 0$ , the equation was simplified to:

$$1 - \frac{IC}{C} \times 100.$$

**Immunization design 1:** Mice were immunized with approximately 500 male or 250 male plus 250 female UV-irradiated cercariae by percutaneous tail exposure. The mice were challenged 4 wk later by exposure to 25 male plus 25 female nonirradiated cercariae.

**Immunization design 2:** Mice were immunized by exposure to 1,000 UV-irradiated cercariae either from individual clones or pools (in all cases, pools were collected from 20 miracidially infected snails), and all were screened simultaneously for their ability to immunize against challenge infection with 50 nonirradiated cercariae from 2 different pools in an attempt to establish whether individual clones were immunogenic, and, if so, whether some clones were better immunogens than others.

**Immunization design 3:** Mice were immunized by exposure to 1,000 UV-irradiated male clone 1 cercariae and challenged by exposure to 50 nonirradiated cercariae of either: (1) males of the same clone and snail passage as the immunizing cercariae (1A), (2) male cercariae of the same clone but another snail passage (1B), or (3) cercariae from a different male clone (3).

## RESULTS

Because we observed that snail infectivity increased during the first few passages, after which it remained constant (Cohen and Eveland, 1984a), all experiments reported here were begun after at least 3 snail passages.

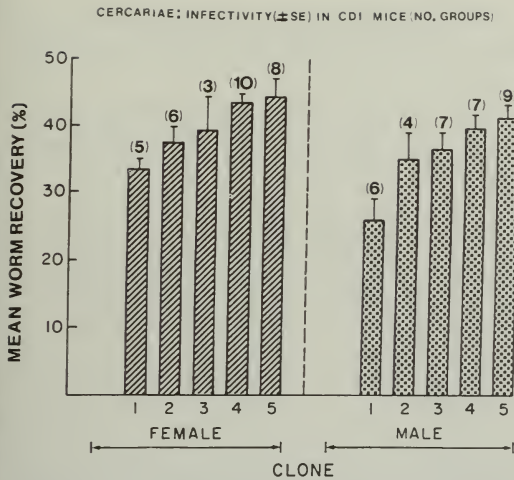


FIGURE 1. Each bar represents the mean percent ( $\pm$ SE) of adult worms recovered by perfusion from 3–10 groups of 5–7 CD-1 mice each 8 wk after infection with 25 male cercariae of 1 clone plus 25 female cercariae from another clone.

#### Infectivity in mice

Mice were exposed to 25 male plus 25 female cercariae to determine the infectivity of cercariae from each clone (Fig. 1). Following exposure, cercarial bodies could not be found on the coverslips, which confirmed that cercarial penetration was complete. The infectivities ranged from 26 to 44%. Although infectivities of some clones overlapped with others, each clone was consistent in its infectivity for mice, regardless of the number of passages through snails, and differences between clones were significant (females,  $P = 0.018$ ; males,  $P = 0.003$ ) using the 1-way analysis of variance (ANOVA). Also, infectivity did not vary significantly within a clone regardless of which cloned partner of the opposite sex was used (Fig. 2) ( $P > 0.30$ , ANOVA).

#### Infectivity in snails

The mean infectivities of 10 clones were determined (Fig. 3). These values are based on 3–10 passages of each clone and 5–25 surviving snails per passage. The infectivities of cloned sporocysts in snails ranged from 44 to 100% and were highly consistent within clones. Differences between clones were highly significant (females,  $P < 0.001$ ; males,  $P < 0.001$ , ANOVA).

#### Cercarial outputs

It was initially anticipated that it would be difficult to detect differences between the cercar-

#### COMPARISON OF INFECTIVITIES OF TWO MALE CLONES IN CD-1 MICE (6 MICE / GROUP)

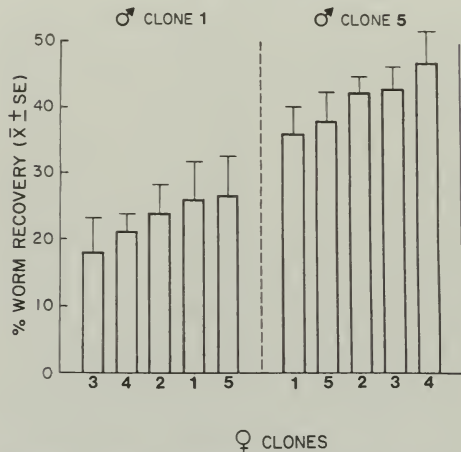


FIGURE 2. Each bar represents the mean male worm recoveries ( $\pm$ SE) from mice infected with 25 male cercariae of male clone A or B plus 25 cercariae of a different female clone.

ial outputs of individual clones due to differences in size and genetic heterogeneity of the snails. However, it soon became apparent that some clones consistently produced more cercariae than others, despite differences in the snail hosts. Therefore the cercarial outputs of individual clones were further examined by measuring the cercarial outputs of individual snails, beginning 6 wk after the onset of cercarial shedding. The mean cercarial output of snails infected with each clone is shown in Figure 4. Cercariae were re-

#### INFECTIVITY OF CLONED SPOROCYSTS IN SNAILS (SURGICALLY INFECTED) ( $\bar{x} \pm$ SE)

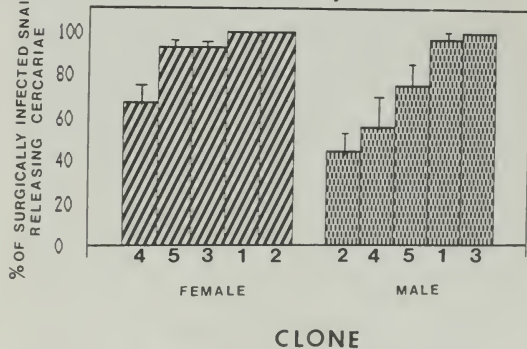


FIGURE 3. Each bar represents the mean infectivity for snails ( $\pm$ SE) of 3–10 passages of sporocysts in groups of 5–25 surgically infected snails that developed patent infections after surviving the prepatent period.



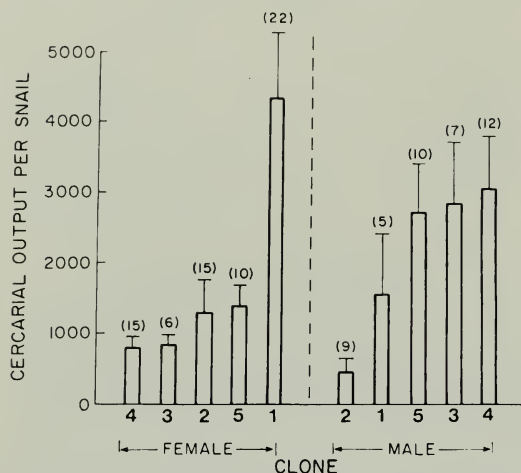
MEAN CERCARIAL OUTPUT ( $\pm$  SE) OF SURGICALLY INFECTED SNAILS (NO. SNAILS)

FIGURE 4. Each bar represents the mean cercarial outputs ( $\pm$  SE) of individual snails surgically infected with different clones 6 wk after the onset of cercarial emergence. Cercariae counts were done for each clone at intervals of 1 wk or more on 7–12 different occasions over a period of approximately 2 yr. There were from 5 to 22 snails producing cercariae from each clone.

covered from individual snails rather than from groups. Cercariae counts were done for each clone at intervals of 1 wk or more on 7–12 different occasions over a period of approximately 2 yr. Each clone had a characteristic cercarial output, and outputs were consistent from passage to passage. Mean cercarial outputs from individual clones ranged from 450 to 4,300 per snail. Differences between both female and male clones were significant ( $P < 0.001$  and  $P < 0.05$ , respectively; ANOVA).

#### UV attenuation of cercariae

We found the duration of the UV irradiation to be as important as the total dose, because more cercariae were attenuated by exposure to 20  $\mu$ watt/cm<sup>2</sup> for 2.0 min than to 40  $\mu$ watt/cm<sup>2</sup> for 1.0 or 1.5 min, even though the latter doses were equal to or greater than the former. When cercariae were exposed to 20  $\mu$ watt/cm<sup>2</sup> for 12 min or more, no worms could be recovered by portal perfusion from mice 6 wk after cercarial exposure (Fig. 5). The dose of 20  $\mu$ watt/cm<sup>2</sup> for 15 min was selected as the standard dose for all subsequent studies. Although it was greater than the minimum dose that prevented worm survival, this relatively high dose allowed a margin of safety against “breakthroughs” because it was

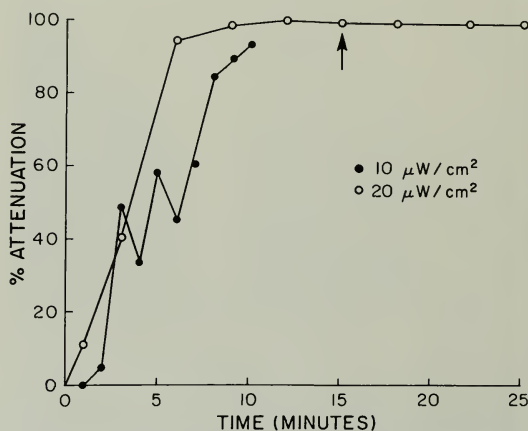
ATTENUATION OF *S. MANSONI* CERCARIAE BY UV EXPOSURE (5 MICE / GROUP)

FIGURE 5. Attenuation of *S. mansoni* cercariae by UV irradiation. Five mice per group were exposed to 500 UV-attenuated cercariae and perfused 6 wk later. Percent attenuation equals 1 – (number of worms recovered from experiment/number of worms recovered from controls)  $\times$  100. (Arrow indicates UV dose selected for the attenuation of cercariae in immunity studies.)

thought that differences in susceptibility to UV irradiation could be a clone-specific trait. Following mouse exposure, very few cercarial bodies were found in the tubes, regardless of UV irradiation dose, indicating that cercarial penetration was essentially complete.

#### Immunization design 1

As shown in Table I, protection was not seen when mice were immunized with 500 irradiated cercariae from male clone 5, then challenged with various clone pairs. However, Table II shows that when mice were immunized with 250 irradiated cercariae of male clone 5 plus 250 irradiated cercariae of female clone 5 and then challenged with various clone pairs, significant protection ( $P < 0.05$ , Student's *t*-test) was seen against male clones 4 and 5. This demonstrated that clones differed in their susceptibilities to immunity, because all of the mice were immunized in the same way. However, it did not demonstrate if a single clone could immunize mice, or whether the failure to immunize was due to the unisexual nature of the immunization, the non-immunogenicity of the specific male clone used, or a requirement for antigenic heterogeneity (i.e., several clones) to induce protection.

TABLE I. Immunization of CD-1 mice with UV-irradiated cloned male cercariae and challenge with clone pairs.\*

Source of challenge cercariae	No. worms/mouse (±SE)	% Protection†	P‡
Male 5	8.7 ± 1.1 (IC) 9.8 ± 1.5 (C)	11.2	N.S.
Female 5	9.3 ± 1.1 (IC) 10.3 ± 1.1 (C)	9.7	N.S.
Male 4	8.5 ± 2.0 (IC) 11.3 ± 1.3 (C)	24.8	N.S.
Female 3	6.8 ± 1.0 (IC) 8.0 ± 1.0 (C)	15.8	N.S.
Male 3	7.9 ± 0.6 (IC) 7.8 ± 1.9 (C)	-1.3	N.S.
Female 1	5.8 ± 0.6 (IC) 5.3 ± 0.6 (C)	-9.4	N.S.

\* Mice immunized with 500 male clone 5 cercariae attenuated by exposure to 20  $\mu$ watt/cm<sup>2</sup> of UV for 15 min. Mice were challenged after 4 wk with 25 male plus 25 female nonirradiated cercariae of the following clone pairs: male 5 + female 5; male 4 + female 3; and male 3 + female 1, respectively.

$$\dagger 1 - \frac{IC - I}{C} \times 100.$$

No worms were recovered from 6 immunization infectivity control mice (I = 0). IC = worm numbers from immunized-challenged mice (experimental; 5–7 mice/group). C = worm numbers of the same sex from challenge infectivity controls (5–7 mice/group).

‡ Probability (Student's *t*-test). Significance of difference from 0% protection. N.S., not significant.

TABLE II. Immunization of CD-1 mice with UV-irradiated male + female cloned cercariae and challenge with homologous and heterologous clone pairs.\*

Source of challenge cercariae	No. worms/mouse (±SE)	% Protection†	P‡
Male 5	5.4 ± 0.5 (IC) 9.8 ± 1.5 (C)	44.9	<0.05
Female 5	7.2 ± 1.0 (IC) 10.3 ± 1.1 (C)	30.1	N.S.
Male 4	6.8 ± 0.6 (IC) 11.3 ± 1.3 (C)	39.8	<0.05
Female 3	7.0 ± 0.4 (IC) 8.0 ± 1.0 (C)	12.5	N.S.
Male 3	7.8 ± 1.9 (IC) 7.8 ± 1.9 (C)	0	N.S.
Female 1	6.2 ± 1.2 (IC) 5.3 ± 0.6 (C)	-17.0	N.S.

\* Mice immunized with 250 male clone 5 plus 250 female clone 5 cercariae attenuated by exposure to 20  $\mu$ watt/cm<sup>2</sup> of UV for 15 min. Mice were challenged after 4 wk with 25 male plus 25 female nonirradiated cercariae of the following clone pairs: male 5 + female 5; male 4 + female 3; and male 3 + female 1, respectively.

$$\dagger 1 - \frac{IC - I}{C} \times 100.$$

No worms were recovered from 6 immunization infectivity control mice (I = 0). IC = worm numbers from immunized-challenged mice (experimental; 5–7 mice/group). C = worm numbers of the same sex from challenge infectivity controls (5–7 mice/group).

‡ Probability (Student's *t*-test). Significance of difference from 0% protection. N.S., not significant.

## Immunization design 2

When mice were immunized with individual clones of cercariae and then challenged with a single pool of cercariae, some of the clones induced significant protection (Student's *t*-test), as shown in Table III. Some clones of either sex appeared to immunize better than others. A 2-way analysis of variance revealed a significant interaction between the susceptibilities of pools A and B ( $P = 0.019$ ). Student's *t*-test also revealed significant differences between pools A and B ( $P < 0.001$ ) in their susceptibilities to immunity against male clone 3 and pool I, but not against the other clones tested (not shown in Table III).

## Immunization design 3

The results are shown in Figure 6. Cercariae from both passages of male clone 1 were strikingly similar in their susceptibility to the immunity induced by attenuated cercariae of the homologous clone. On the other hand, the cercariae of male clone 3 were significantly more susceptible ( $P < 0.001$ , ANOVA) than cercariae of clone 1. These results demonstrated that some clones were more susceptible to immunity than others. They further showed that homologous

immunity was not necessarily stronger than heterologous immunity.

## DISCUSSION

For all of the traits examined, consistency within clones and significant differences between them were detected. These results demonstrate clearly that variability in PR-1 *Schistosoma mansoni* is due to differences between clones rather than to phenotypic plasticity of a uniform genetic background, because these studies were done under standardized conditions. Clones maintained by microsurgical transplantation did not vary phenotypically as a function of time or subpassaging through snails, showing that genetic heterogeneity in the schistosomes is an important phenomenon that probably contributes greatly to variable experimental results.

The infectivity of each clone for the M line *Biomphalaria glabrata* stock used in our laboratory and for CD-1 mice was determined, and infectivities did not appear to differ with time after the first few passages. In mice, male or female clone infectivity was not influenced by the cloned partner of the opposite sex that was used for coinfection. It seems likely that the isolation of further clones will result in the detection of



TABLE III. Immunization of CD-1 mice with UV-irradiated cloned or pooled cercariae against challenge with pooled cercariae.\*

Source of immunizing cercariae	Source of challenge cercariae	No. worms/mouse ( $\pm$ SE)	% Protection†	P‡
None	Pool A	19.8 $\pm$ 1.7 (C)	—	—
None	Pool B	19.3 $\pm$ 3.0 (C)	—	—
Male 4	Pool A	8.8 $\pm$ 1.2 (IC)	55.6	<0.001
Male 4	Pool B	12.3 $\pm$ 2.0 (IC)	36.2	N.S.
Male 3	Pool A	17.6 $\pm$ 1.2 (IC)	11.1	N.S.
Male 3	Pool B	9.7 $\pm$ 1.8 (IC)	49.7	<0.02
Male 1	Pool A	9.2 $\pm$ 1.3 (IC)	53.5	<0.002
Male 1	Pool B	13.0 $\pm$ 3.1 (IC)	32.6	N.S.
Female 5	Pool A	12.7 $\pm$ 4.2 (IC)	35.9	N.S.
Female 5	Pool B	14.0 $\pm$ 1.5 (IC)	27.5	N.S.
Female 3	Pool A	12.8 $\pm$ 1.9 (IC)	35.4	<0.05
Female 3	Pool B	12.5 $\pm$ 1.4 (IC)	35.2	<0.05
Pool I	Pool A	6.4 $\pm$ 1.3 (IC)	67.7	<0.001
Pool I	Pool B	12.7 $\pm$ 1.2 (IC)	34.2	N.S.

\* Mice immunized with 1,000 cloned or pooled (collected from 20 miracidially infected snails) cercariae attenuated by exposure to 20  $\mu$ watt/cm<sup>2</sup> of UV for 15 min. Mice were challenged 4 wk later with 50 non-irradiated pooled cercariae collected from 20 miracidially infected snails.

$$\dagger 1 - \frac{IC - I}{C} \times 100.$$

No worms were recovered from 5 to 7 immunization infectivity control mice per group (i.e., I = 0). C = worm numbers from challenge infectivity controls (5–7 mice/group). IC = worm numbers from immunized-challenged mice (experimental); 5–7 mice/group).

‡ Probability (Student's *t*-test). Significance of difference from 0% protection. N.S., not significant.

some with even more divergent infectivities, and it may be possible to isolate clones that are unable to develop to maturity in mice and then test them as attenuated vaccines. Obviously, such clones could be propagated only by microsurgical transplantation.

Overall, the cercarial outputs of surgically and miracidially infected snails were comparable. Some clones were distinctly inferior to others in their cercarial outputs (e.g., Fig. 4, male 2), and this was observed for many passages. Interestingly, there was a clear separation between the ability of sporocysts to multiply within snails (i.e., infectivity for snails) and their cercarial production (e.g., Figs. 3 and 4, male 1). When the entire population of snails infected with a particular clone was examined, there was some variation in cercarial output between snails, probably due to differences in snail size and genetic heterogeneity in snails. This variability was insignificant compared to the striking differences between clones, and those differences were consistent.

Cloning offers numerous possibilities for research. For example, after an experiment has been done, genetically identical organisms remain

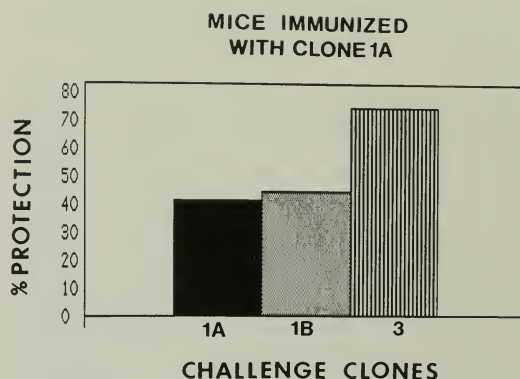


FIGURE 6. Immunization of mice (5–7 mice/group) with 1,000 cloned or pooled UV-irradiated male clone 1A cercariae by percutaneous tail exposure, followed 4 wk later by exposure to 50 nonirradiated cercariae of either: (1) males of the same clone and passage as the immunizing cercariae (1A), (2) male cercariae of the same clone but another passage (1B), or (3) cercariae from a different male clone (3).

available for further experiments. It will therefore be possible to study biochemical and antigenic differences between clones that differ in their immunogenicity, susceptibility to immunity, or drug sensitivity, and determine which properties correlate with the biological attributes. It is also important to compare the various *in vitro* and *in vivo* models of schistosome immunity using cloned organisms, because genetic differences between pools of cercariae would be eliminated as a possible explanation for failure to find a correlation. The applicability of the cloning technology is also greatly enhanced by the ability to cryopreserve clones (Cohen and Eveland, 1984b).

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## SPONTANEOUS FLIGHT ACTIVITY OF *AEDES TRIVITTATUS* INFECTED WITH *DIROFILARIA IMMITIS*

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**ABSTRACT:** Spontaneous flight activity of *Dirofilaria immitis*-infected *Aedes trivittatus* was evaluated by using an acoustic activity system. The activity of mosquitoes infected with low numbers of filarial larvae (1-4) was similar to that of uninfected mosquitoes. However, mosquitoes infected with more than 4 larvae became more active than uninfected mosquitoes 8 days after infection. Peak flight activity (circadian) occurred at the same time in both infected and uninfected mosquitoes, but infected mosquitoes were much more active during normal periods of quiescence. Flight activity of mosquitoes infected with more than 4 larvae was suppressed on days 10 and 14 postinfection, corresponding to times of greatest disruption of the Malpighian tubules by the developing larvae.

*Aedes trivittatus* (Coquillett) is a natural vector of *Dirofilaria immitis* (Leidy) in Iowa (Christensen and Andrews, 1976; Christensen, 1977a, 1977b) and Indiana (Pinger, 1982). *Aedes trivittatus* is a floodwater mosquito with a biology similar to that of *Aedes vexans* Meigan. In Iowa, *A. trivittatus* is locally abundant and a persistent pest of humans. Dogs were identified as a common host of *A. trivittatus* in Indiana (Nasci, 1984), indicating that this mosquito species contacts *D. immitis* under natural circumstances.

Developing *D. immitis* larvae caused increased mortality of *Aedes aegypti* (L.) (Kershaw et al., 1953), *Aedes sollicitans* (Walker) (Beam, 1966), and *A. trivittatus* (Christensen, 1978). In all cases, mortality was higher in more heavily infected mosquitoes. Additionally, *A. aegypti* infected with *D. immitis* were less active than uninfected counterparts (Berry et al., 1987a).

This study was designed to determine if developing *D. immitis* larvae alter the spontaneous flight activity and flight behavior of *A. trivittatus*, a proven natural vector. A computer-interfaced acoustic activity system (Rowley et al., 1987) was used to evaluate flight activity. The parameters investigated included monitoring the circadian activity patterns of mosquitoes with different parasite burdens, flying time, the number of flights initiated, and an activity score.

### MATERIALS AND METHODS

*Aedes trivittatus* were reared from eggs laid by field-collected females following the protocol described by Christensen and Rowley (1978). Adult *D. immitis* were

maintained in beagle dogs. Mosquitoes were infected by feeding on a water-jacketed membrane feeder (Rutledge et al., 1964) containing dog blood with 94 and 168 microfilariae/20  $\mu$ l for experiments 1 and 2, respectively. Before being filled, the membrane feeder was fitted with a freshly prepared mouse skin through which mosquitoes could feed. Control mosquitoes were given a blood meal without microfilariae.

On day 3 postinfection (PI), 32 mosquitoes (24 infected and 8 uninfected in experiment 1; 16 infected and 16 uninfected in experiment 2) were placed individually in acoustic chambers made from modified reagent bottles (Jones et al., 1967). Bottoms of the bottles were replaced with tightly stretched filter paper and a tight layer of plastic wrap. A 1.5-ml microcentrifuge tube, filled with absorbent cotton saturated in 0.3 M sucrose, was suspended from the mouth of each chamber as a source of both carbohydrate and moisture. The photocycle consisted of 16 hr of light, followed by 8 hr of darkness, with a sharp transition between light and dark. Mosquitoes were randomly assigned to chambers and allowed 1 day to acclimate. The number of flights, flying time, and an activity score were recorded for each mosquito in each 30-min period. The activity score represented the number of minutes within a period during which a mosquito made at least 1 flight of any duration (Jones et al., 1967).

Activity chambers were examined at least twice daily. Any dead mosquitoes were replaced with mosquitoes of identical age and infection category. Replacement mosquitoes were allowed at least 24 hr to acclimate before being included in the analysis. Flight activity was monitored on days 4-15 PI. The temperature during the experiment was  $27 \pm 1$  C.

The number of *D. immitis* larvae in each mosquito was determined by dissecting the mosquito in *Aedes* saline (Hayes, 1953). Dead mosquitoes were dissected immediately upon removal from chambers. At the end of an experiment, all mosquitoes were anesthetized by refrigeration and dissected. Developing larvae in the Malpighian tubules and infective larvae in the head capsule or hemocoel were counted to determine the parasite intensity for each mosquito.

Mosquitoes were grouped according to parasite intensity. Statistical analysis consisted of analysis of variance of the daily activity score, number of flights, and flying time for each day PI. Daily activity patterns and

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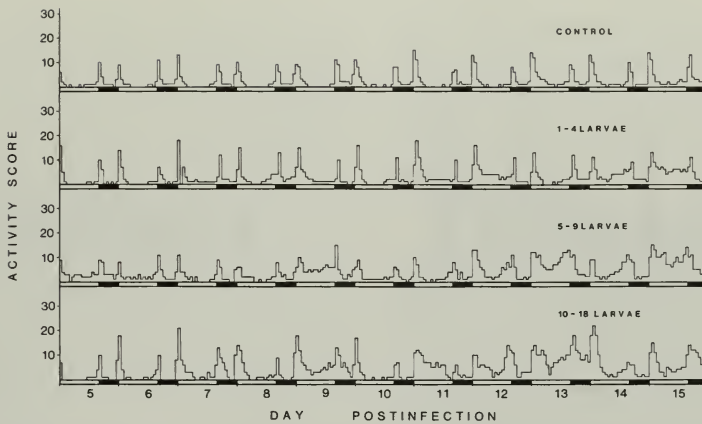


FIGURE 1. Spontaneous flight activity (circadian) of *Aedes trivittatus* infected with different intensities of *Dirofilaria immitis* on days 4–15 postinfection. Mosquitoes were maintained on a 16L:8D photocycle.

changes produced by different parasite burdens were compared. Differences were considered significant at  $P < 0.05$ . Dead mosquitoes were deleted from the analysis on the day they died, and replacement mosquitoes were not included on the following day.

## RESULTS

*Dirofilaria immitis* strongly influenced spontaneous flight activity of *A. trivittatus* (Fig. 1). Uninfected mosquitoes had a large peak of flight activity beginning at the onset of the photophase and lasting for 4 hr. A second, smaller period of activity occurred at the beginning of the scotophase. Uninfected mosquitoes were inactive through most of the scotophase. Mosquitoes harboring developing larvae had the same pattern of flight activity as uninfected counterparts early in the infection. However, as filarial worms developed (after day 10), activity of infected mosquitoes continued throughout the photophase. Additionally, infected mosquitoes were active throughout the scotophase, but at a much lower level.

Before day 8 PI, infected mosquitoes were as active as control mosquitoes (Fig. 2a). After day 8, mosquitoes infected with more than 4 filarial larvae were significantly more active than uninfected mosquitoes on most days. Though consistently higher, activity scores of lightly infected (1–4 larvae) mosquitoes were not significantly different from those of control mosquitoes throughout the experiment. Daily activity scores of mosquitoes infected with 5 or more filarial larvae were as much as 3 times higher than those of uninfected mosquitoes. Much of the increase in total flight activity occurred during periods

outside the times when the mosquitoes were normally active. However, activity of infected mosquitoes was equivalent to that of uninfected mosquitoes on day 10 PI. On day 14 PI, flight activity of mosquitoes infected with 5 or more larvae was markedly reduced as compared to the level of activity on day 13 or day 15 PI.

Differences in the number of flights made by mosquitoes in each group were similar to the differences in activity scores (Fig. 2b). The number of flights made by individual mosquitoes increased through the experiment in all groups. Mosquitoes infected with 5 or more filarial larvae made more flights than uninfected mosquitoes, but not until after day 8 PI. Infected mosquitoes made fewer flights on day 10 and again on day 14 PI. This change in spontaneous activity is reflected in the activity scores for these days (Fig. 2a).

Infected mosquitoes spent more time flying than uninfected mosquitoes (Fig. 2c). However, the differences were not as great as the differences noted in the number of times a mosquito actually initiated a spontaneous flight. This indicated that infected mosquitoes made more, but shorter flights (Fig. 2d). The time that infected mosquitoes spent flying decreased markedly on day 10 and again on day 14 PI, especially in mosquitoes harboring 5 or more developing larvae. The mean length of individual flights made by uninfected mosquitoes was 9–10 sec on most days. Mosquitoes infected with fewer than 5 *D. immitis* larvae made flights of similar duration (9–10 sec) throughout the experiment. Individual flights made by more heavily infected mosquitoes were of shorter duration after day 8 in moderately



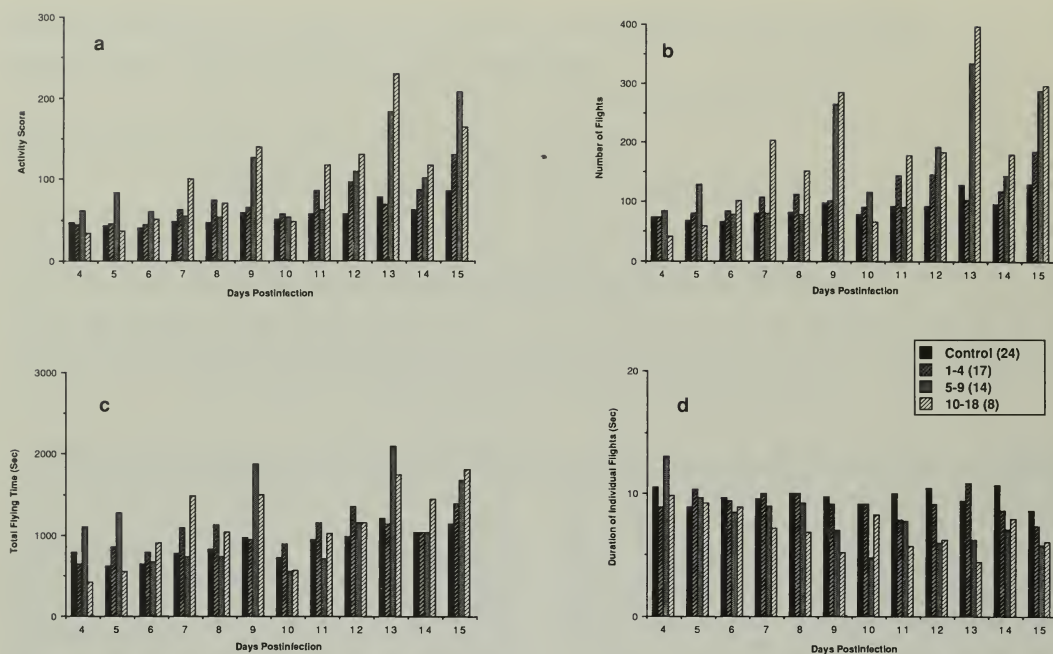


FIGURE 2. Flight activity of *Aedes trivittatus* infected with different intensities of *Dirofilaria immitis* on days 4–15 postinfection: (a) activity scores; (b) number of flights; (c) total flying time; (d) duration of individual flights. The numbers in parentheses indicate the number of female mosquitoes in each category.

infected (5–9 larvae) mosquitoes and after day 6 in heavily infected (10–18 larvae) mosquitoes.

### DISCUSSION

Developing filarial worms have a pronounced effect on the spontaneous flight activity and flight ability of mosquito hosts. Larvae of *Brugia pahangi*, developing in the flight muscle of *A. aegypti*, cause a loss of flight ability (Townson, 1970; Paige and Craig, 1975). Hockmeyer et al. (1975) reported that *B. pahangi*-infected *A. aegypti* flew significantly less than uninfected mosquitoes and that a greater number of infected mosquitoes died or were unable to fly. Husain and Kershaw (1971) found that *B. malayi* had a similar effect on the flight ability of *Aedes togoi* (Theobald). Developing *B. pahangi* and *D. immitis* also cause a reduction in the circadian flight activity of infected *A. aegypti* (Berry et al., 1986, 1987a; Rowland and Lindsay, 1986). All previous studies found that developing filarial larvae reduced flight ability or flight activity of host mosquitoes. In this study, *D. immitis* stimulated flight activity of *A. trivittatus*. *Aedes trivittatus* is a natural vector of dog heartworm and the effect of the parasite on this species is markedly different from that observed in the experimental vector, *A. aegypti*.

The circadian pattern of flight activity of uninfected *A. trivittatus* was similar to that seen in previous laboratory studies using an identical light regime (Berry et al., 1987b). The bimodal circadian activity pattern consisted of a peak of flight activity at lights-on and a smaller peak at lights-off. Field studies have indicated that these are the times of peak activity of *A. trivittatus* (Thompson and Dicke, 1965; Wright and Knight, 1966). Beginning on day 9 PI, infected mosquitoes remained active throughout the photophase, and some activity was maintained during the scotophase. The principal peaks (as seen in the controls) were evident, but flight activity continued through periods when this species is normally inactive. In contrast, *A. aegypti*, infected with *D. immitis*, were less active than uninfected controls, but the timing of activity was identical in infected and uninfected mosquitoes (Berry et al., 1987a).

Total flight activity of *A. trivittatus* increased slightly during the experiment. A similar trend was reported in a previous laboratory study (Berry et al., 1987b). The mean duration of each flight remained the same in uninfected mosquitoes, so the trend was evident in all variables. Activity scores and number of flights of the infected mosquitoes increased during the experiment, but the

length of individual flights decreased. Consequently, an increase in the number of times a mosquito flew did not lead to proportionately greater amounts of time spent flying. Peterson (1980) found that activity scores, number of flights, and flying time tend to yield similar hourly patterns of flight activity. In this study, the duration of individual flights made by mosquitoes infected with 5 or more larvae was shorter than that of uninfected mosquitoes. The pattern for each variable was different. In previous studies, filarial worms, *B. pahangi* or *D. immitis*, caused *A. aegypti* to make shorter flights (Berry et al., 1986, 1987a).

Flight activity of infected mosquitoes was sharply depressed on days 10 and 14 PI. Christensen and Hollander (1978) found that second- and third-stage *D. immitis* first appear on days 8 and 11 PI, respectively, when infected *A. trivittatus* are held at 26.5°C. The majority of first-stage larvae molt to the second stage between days 8 and 10, and third-stage larvae appear on days 11–14 PI (Christensen, 1977b). Similar developmental periods have been reported for *D. immitis* in *Aedes triseriatus* (Say) and *A. vexans* (Fortin and Slocombe, 1981). Palmer et al. (1986) found extensive damage to Malpighian tubule cells of *D. immitis*-infected mosquitoes by days 8–10 PI. The activity of infected mosquitoes was reduced at times corresponding to specific periods in the development of filarial larvae. Reduced flight activity on day 10 PI corresponded with the molt from first to second stage, and the decrease in activity on day 14 corresponded with the time when infective larvae migrated to the mouthparts, even though many larvae were still in the Malpighian tubules at that time. In comparison, flight activity of *A. aegypti* was suppressed when *B. pahangi* molted (Berry et al., 1986), but not when *D. immitis* molted (Berry et al., 1987a).

Increased flight activity of infected mosquitoes could be associated with an irritation related to the activity of the larvae. Alternatively, activity might be stimulated by a reduction in the function of the Malpighian tubules. The periods of reduced activity might be caused by release of waste materials generated by the filarial larvae into the mosquito.

Increased flight activity undoubtedly enhances the possibility that the mosquito will contact a new host. Flight activity outside normal periods of activity also might enhance contact with host animals. Differences in the influence of *D. im-*

*mitis* on flight activity of *A. aegypti* as compared with *A. trivittatus* probably are related to the natural relationship between *A. trivittatus* and *D. immitis*, indicating that natural hosts might be influenced differently than experimental vector species.

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## SIZE OF INOCULUM DOSE REGULATES IN PART WORM BURDENS, FECUNDITY, AND LENGTHS IN OVINE *HAEMONCHUS CONTORTUS* INFECTIONS\*

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**ABSTRACT:** Density-dependent factors frequently have been shown to regulate population parameters of free-living and parasitic helminths. To test the effects of various infection levels of *Haemonchus contortus* on fecundity and worm size, lambs were inoculated with 3,000, 10,000, and 30,000 infective larvae. Daily eggs per gram (epg) and daily total fecal production per lamb were monitored continuously. Worms were collected from abomasa on 6, 15, 22, and 30 days postinfection (PI). Female worms were smaller on each day in the high-dose group when compared to female worms from the low-dose group; males in the high-dose group were smaller from days 15 through 30 PI. The high- and medium-dose groups had higher mortality rates on day 30 PI, and fecundity (eggs/female/day) was 78% lower. Daily epg and daily total eggs/lamb/day were lower in the high-dose group. Fecundity and worm size were correlated with the log-transformed dose level but not with adult worm number. Early parasite and/or host responses apparently exert long-term negative effects on growth and reproduction relative to the size of the establishing population of *H. contortus*.

The density of helminths frequently alters population parameters (reviewed by Keymer, 1982). The best-defined system is the free-living nematode *Caenorhabditis elegans*, in which the gene for a dauer-larvae-inducing pheromone has been identified (Riddle, 1986). At high concentrations of pheromone, i.e., high larvae concentrations, the formation of the developmentally arrested dauer larval stage is enhanced. In parasitic helminths, putative "crowding factors" inhibited the growth of the cestode *Hymenolepis diminuta*, apparently through blockage of the cell cycle (Roberts and Insler, 1982).

In parasitic nematodes, Swanson and Bone (1983) found a significant positive correlation of daily egg production with number of *Nippostrongylus brasiliensis* in female murine hosts, but not in male hosts. Michel (1963) proposed that egg production of *Ostertagia ostertagi* in calves is stereotypic irrespective of worm burdens; fecal egg counts were similar in all calves irrespective of inoculum dose or mode of administration in experiments based solely on eggs per gram (epg) counts. Density-dependent growth rates correlated significantly with egg concentration and weight of adult *Haemonchus contortus* (Le-

Jambre et al., 1971, and Ractliffe et al., 1971, respectively).

This study was designed to determine an inoculum level of infective larvae of *H. contortus* that would maximize fecal egg production from source lambs. To test the hypothesis proposed by Michel (1963) that density-dependent factors regulate egg production, parasite numbers, lengths, and fecundity of populations were measured.

### MATERIALS AND METHODS

Helminth-free Polled Dorset ewe and wether lambs (4-5 mo old) were inoculated with 3,000 (low dose,  $n = 19$ ), 10,000 (medium dose,  $n = 5$ ), or 30,000 (high dose,  $n = 17$ ) infective larvae of the BPL strain of *Haemonchus contortus*. Lambs were maintained individually in cages with suspended screens to collect feces. Lambs from the low and high groups were killed ( $n = 4, 4$ , and  $3$ /dose/day) on days 6, 15, and 22 post-inoculation (PI), respectively. The abomasa were removed, and the contents rinsed and filtered with physiological saline. Contents were fixed with 5% buffered formalin. Larvae were stained with Lugol's iodine, and larvae and adults were magnified (10-30 $\times$ ) and projected onto a digitizing screen; lengths were measured with a computer-assisted program (R & M Biometrics, Nashville, Tennessee).

From day 21 PI, daily egg counts were measured from the feces of the remaining lambs, and total daily fecal production was weighed for each lamb. On day 30 PI, these lambs ( $n = 8, 5$ , and  $6$ , respectively) were killed, and their abomasal contents were processed as described previously. Fecundity (eggs/female/day) was calculated by averaging the final 3 days of total egg production/lamb and dividing by the number of females recovered at postmortem.

All analyses were computed with SAS programs (SAS

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TABLE I. *Lengths (mm, n = 30/sex/dose,  $\bar{x} \pm SEM$ ) of Haemonchus contortus larvae and adults periodically recovered after inoculation of lambs. Means in each row with different superscripts are significantly different ( $P < 0.05$ ).*

Days post-inoculation	Inoculum	
	3,000	30,000
6		
Male	3.89 $\pm$ 0.06 <sup>a</sup>	3.80 $\pm$ 0.03 <sup>a</sup>
Female	4.51 $\pm$ 0.05 <sup>a</sup>	3.89 $\pm$ 0.04 <sup>b</sup>
No. lambs	4	4
15		
Male	12.83 $\pm$ 0.09 <sup>a</sup>	10.34 $\pm$ 0.15 <sup>b</sup>
Female	17.22 $\pm$ 0.20 <sup>a</sup>	14.06 $\pm$ 0.28 <sup>b</sup>
No. lambs	4	4
22		
Male	13.09 $\pm$ 0.14 <sup>a</sup>	10.89 $\pm$ 0.21 <sup>b</sup>
Female	15.75 $\pm$ 0.28 <sup>a</sup>	11.30 $\pm$ 0.34 <sup>b</sup>
No. lambs	3	3

Institute, Cary, North Carolina). Inoculum doses, fecal egg counts, and total daily egg production/lamb were transformed to  $\log_{10}$  before analyses to normalize the distribution. Fecal egg counts and total daily egg production (epg  $\times$  total daily fecal production/lamb) were analyzed by general linear models that recognized repeated sampling within an experimental unit. Treatments were compared with Waller/Duncan's multiple range tests. Lengths and number of worms were compared by analysis of variance; means were compared by Duncan's multiple range tests. Correlations of adult worm lengths and fecundity with total worm populations as well as with log-transformed inoculum doses were performed with the general linear model. Differences were considered significantly different at  $P < 0.05$ .

## RESULTS

The methods used for screening abomasal contents did not permit accurate total recoveries of larval and young adult populations on days 6, 15, and 22 PI. However, as early as day 6 PI,

fourth-stage larval females were smaller (14%) in the high inoculum dose (Table I). This stunting of females increased on days 15 (19%) and 22 PI (29%). Male larvae in both groups were similar on day 6 PI, but high-dose males were smaller on days 15 (19%) and 22 PI (17%) (Table I).

Lengths of mature adult males and females were progressively smaller with increasing inoculum dose on day 30 PI (Table II). These reductions in sizes were correlated significantly with the log-transformed inoculum doses ( $r = 0.68$ ,  $r = 0.56$ , respectively), but lengths were not correlated with final worm populations. Population sizes were similar among the low- and medium-dose groups (Table II). In contrast, the high-dose group had more males and females, but the fecundity was significantly reduced (78%) in the high-dose group. The numbers of worms were positively correlated with the log-transformed inoculum dose ( $r = 0.56$ ).

The profiles of daily epg and total daily egg production were similar among the groups; however, the magnitude of the curves of the high-dose group were significantly lower when compared to the other groups (Figs. 1, 2). Fecundity (Table II, eggs/female/day) was not correlated with number of females or total number of worms but was significantly correlated with log-transformed inoculum dose ( $r = 0.49$ ).

## DISCUSSION

Increasing the size of the inoculum over the range utilized herein increased the worm burden of *H. contortus*. However, compensatory reduction in fecundity actually decreased the net egg production. In contrast to Michel's (1963) results, these data indicate that the fecundity and worm length depend on the magnitude of the

TABLE II. *Haemonchus contortus* worm populations, sizes, and egg production ( $\bar{x} \pm SEM$ ) from lambs infected with inocula of 1 of 3 doses on day 30 postinoculation. Means with different superscripts in the same row are significantly different ( $P < 0.05$ ). Numbers in parentheses are sample sizes.

	Inoculum dose		
	3,000	10,000	30,000
No. lambs	8	5	6
No. males	678 $\pm$ 115 <sup>a</sup>	907 $\pm$ 397 <sup>a</sup>	2,667 $\pm$ 857 <sup>b</sup>
No. females	722 $\pm$ 116 <sup>a</sup>	960 $\pm$ 483 <sup>a</sup>	2,901 $\pm$ 857 <sup>b</sup>
No. adults	1,421 $\pm$ 237 <sup>a</sup>	1,814 $\pm$ 875 <sup>a</sup>	5,568 $\pm$ 1,740 <sup>b</sup>
% Inoculum recovered	47.3 <sup>a</sup>	18.1 <sup>b</sup>	18.6 <sup>b</sup>
Mortality rate - $\ln(\text{adults/inoculum})/30$ days	0.0249	0.0569	0.0561
Eggs/female/day	3,496 $\pm$ 802 <sup>a</sup>	4,180 $\pm$ 738 <sup>a</sup>	855 $\pm$ 323 <sup>b</sup>
Length of males (mm)	16.3 $\pm$ 0.1 <sup>a</sup> (799)	15.7 $\pm$ 0.1 <sup>b</sup> (437)	15.0 $\pm$ 0.1 <sup>c</sup> (600)
Length of females (mm)	23.1 $\pm$ 0.2 <sup>a</sup> (144)	20.7 $\pm$ 0.3 <sup>b</sup> (71)	20.3 $\pm$ 0.2 <sup>c</sup> (123)

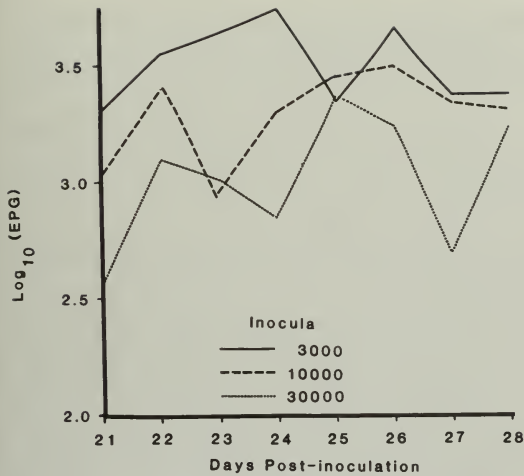


FIGURE 1. Log-transformed profiles of daily eggs per gram (epg) from lambs given 1 of 3 inoculum doses of infective larvae of *Haemonchus contortus* on day 0.

initial inoculum and not on the resultant worm burden. Therefore, these relationships suggest that the early postinoculation responses of the parasite and/or the host negatively altered the growth and reproductive capabilities of the mature adult worms. Possibly, a "crowding factor" released during early establishment of the infection could alter growth and development as in *H. diminuta* (Roberts and Insler, 1982) or *C. elegans* (Riddle, 1986).

Conversely, the host might be responding rapidly biochemically and/or immunologically in a dose-dependent manner to the larvae. That such rapid responses are possible is demonstrated by the work of Honde et al. (1985), who found that serum levels of gastrin and somatostatin increased within minutes after inoculation of *H. contortus*. Exposure to abnormal levels of host products during a critical developmental stage might alter subsequent growth. Because less than half of the variation ( $r^2$ ) was attributable to inoculum size, other undefined factors also must regulate fecundity and growth in *H. contortus* infections. Fecundity was also negatively correlated with size of inoculum dose in ovine *Nematodirus spathiger* (Donald et al., 1964), in ovine *Trichostrongylus axei* (Kates and Turner, 1960), in lapine *Obeliscoides cuniculi* (Russell et al., 1966), and in bovine *O. ostertagi* and *Cooperia oncophora* infections (Michel, 1963, and Herlich, 1965, respectively).

The earliest time point sampled, 6 days PI, demonstrated that a significant size reduction in the length of female larvae already had occurred,

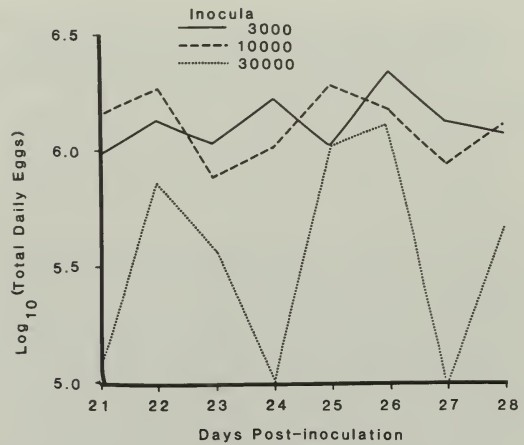


FIGURE 2. Log-transformed profiles of total daily egg production/lamb (eggs per gram  $\times$  total daily fecal production) from lambs given 1 of 3 inoculum doses of infective larvae of *Haemonchus contortus* on day 0.

reflecting the influence of early establishment density on the growth of the larvae. The increase in the degree of stunting through day 22 PI suggests the continued production of a putative "crowding factor" or a sustained host response. Similarly, Anderson and Michel (1977) suggested that the survival pattern of adult *O. ostertagi* was determined at the start of infection by the size of the larval inoculum.

These results have particular implications for the interpretation of experiments or surveys in which infection levels are inferred from fecal egg counts, i.e., pasture contamination studies. Dense concentrations of infective larvae on pasture might not positively correlate with parasite worm burdens or nematode egg production as is often assumed.

#### ACKNOWLEDGMENT

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## INDUCTION OF MACROPHAGE MOTILITY BY A T-CELL LINE FROM BALB/C MICE SPECIFIC FOR *PLASMODIUM BERGHEI* MALARIA

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**ABSTRACT:** A long-term antimalaria T-cell line (AMTL) expressing a helper phenotype (Thy 1.2<sup>+</sup>, Lyt 2.2<sup>-</sup>) was established from *Plasmodium berghei*-recovered Balb/c mice. The ability of this T-line to induce macrophage motility was measured *in vivo* and *in vitro*. Adoptive transfer of AMTL cells to normal Balb/c mice showed an increased delayed hypersensitivity response to the homologous antigen, i.e., parasitized erythrocytes (PE). *In vitro*, AMTL culture supernatant (AMTL-SUP) augmented chemotactic locomotion of macrophages derived from both normal and infected mice. However, the effect on normal macrophages was significantly higher. AMTL cells adoptively transferred to normal mice had no effect on parasitemia levels or mortality rate after subsequent infection with *P. berghei*.

Partial characterization of the AMTL-SUP indicated the involvement of a protein of about 12,600 Daltons in the enhancement of chemotaxis. These findings suggest that the AMTL cells and chemoattractants produced by them can induce macrophage motility, and that the macrophage malfunction in Balb/c with *P. berghei* infection is not due to defects at the T-lymphocyte level.

Cell-mediated immunity plays an important role in the development and maintenance of protection against malaria infection. The massive T-cell activation (Jayawardena et al., 1975) along with the increase in parasitemia and mortality in thymus-deprived mice (Weidanz and Rank, 1975) strongly suggest a crucial role for regulator and effector T-cells in the malaria infected host. T-cells activated by antigens or mitogens respond by releasing activating factors that stimulate macrophages to increase their metabolic, secretory, and functional activity (David, 1975; Ladzins et al., 1978). Hydrogen peroxide production, interleukin-1 secretion, and chemotactic activity are only a few of many activities known to be affected by T-cell activation.

There is evidence that protection in malaria-infected animals is related to the interaction of T-cells with macrophages and to the release of macrophage-derived factors toxic or inhibitory to the parasites (Allison and Eugui, 1983). It is also known that mice infected with malaria parasites have severe impairment of humoral and cellular immune responses (Greenwood et al., 1971). It was shown that antibody formation, delayed hypersensitivity to unrelated antigens (Taverne et al., 1971), and *in vitro* proliferative response to mitogens (Lelchuk et al., 1984) are depressed. Several alterations have been demonstrated at the macrophage level: the capacity

to detoxify endotoxin by macrophages of infected animals was markedly reduced (Loose and DiLuzio, 1976), processing and presentation of thymus-dependent antigens was aberrant (Weidanz and Rank, 1975; Warren and Weidanz, 1976), and macrophages from malaria-infected mice were unable to kill facultative intracellular bacteria (Murphy, 1981).

Because the immune response to malaria parasites is a complex event that involves T-cells and macrophages, the use of pure T-lymphocyte populations is helpful in clarifying the mechanism of the interaction between these populations. In the present work we established a long-term proliferative antimalaria T-cell line from convalescent mice, and studied the capacity of these cells and their supernatants to stimulate macrophage motility *in vivo* and *in vitro*.

### MATERIALS AND METHODS

#### Animals

Female Balb/c mice, 6-8 wk old, were obtained from the Hebrew University-Hadassah Medical School animal facility.

#### Immunization

Mice were infected intraperitoneally with 10<sup>6</sup> parasitized erythrocytes (PE) of the lethal strain *Plasmodium berghei* (NYU-2). After 10 days, when parasitemia levels reached a value of 20-35%, the mice were treated with chloroquine (Sigma, U.S.A.), 300 mg/L tap water administered orally for a further 10 days. At the end of the treatment period no parasites were detected in the peripheral blood, and the animals recovered from a challenge of 2 × 10<sup>6</sup> PE given 30 days after infection.

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### Antigen preparation

The antigen used was a highly enriched preparation of parasitized erythrocytes (PE) (90–95%). PE obtained from infected mice were prepared using 1.075–1.080 density Percoll (Pharmacia, Sweden) as described by Nillni et al. (1981). Normal erythrocytes (NE) were used as controls.

### Establishment of an *in vitro* proliferative antimalaria T-cell line (AMTL)

Immune mice were killed 5–8 days after the second challenge with PE. Spleens were removed, passed through a 60-mesh sieve, and incubated for 20 min with 0.87% ammonium chloride buffered with  $\text{Tris-NH}_4\text{Cl}$  in order to hemolyze red blood cells. White cells were washed, counted, and adjusted to  $5 \times 10^6$  cells/ml RPMI-1640 (GIBCO, U.S.A.) containing 5% heat-inactivated fetal calf serum (FCS; Difco, Detroit, Michigan). Tissue culture dishes ( $90 \times 20$  cm, Nunc, Denmark) containing  $25 \times 10^6$  spleen cells and  $3 \times 10^6$  PE were incubated at 37°C in a humidified incubator with 5%  $\text{CO}_2$ . After 6 days, cells were harvested, centrifuged, and resuspended in growth medium (GM) containing RPMI-1640 supplemented with 1 mM glutamine, 100  $\mu\text{g}/\text{ml}$  streptomycin, 100 U/ml penicillin, 1 mM sodium pyruvate, 1 mM nonessential amino acids,  $5 \times 10^{-5}$  M mercaptoethanol (Bio-Lab, Israel), 1 mM HEPES (Sigma, U.S.A.), 10% FCS, and 25% interleukin-2 (IL-2) containing supernatant from concanavalin-A-stimulated rat spleen cells, prepared as described by Gross et al. (1984). Alpha-methyl mannoside (20 mg/ml) was added to the IL-2-containing supernatant to abolish the Con A activity. GM was changed every 2–3 days, according to the cell proliferation rate.

### Restimulation of the AMTL cells

Restimulation was performed every 2–4 wk. Irradiated (3,000 rad) syngeneic spleen cells ( $60 \times 10^6$ ) and PE ( $1 \times 10^6$ ) were added to  $2 \times 10^6$  AMTL cells in a final volume of 10 ml.

### Control lines

Two control lines were established:

1) Anti-purified protein derivative of tuberculin (PPD) T-cell line (anti-PPD-TL) was prepared from Balb/c mice injected subcutaneously with 0.3 ml Freund's complete adjuvant (Difco) 14 days prior to splenectomy. For establishment of the anti-PPD-TL,  $50 \times 10^6$  spleen cells were supplemented with 250 g PPD (Statens, Denmark) and 3 days later transferred to GM. Fifty  $\mu\text{g}$  of PPD per  $2 \times 10^6$  cells were used as antigen for restimulating the anti-PPD-TL.

2) Anti-*Leishmania* T-cell line (anti-LTL) was prepared from peripheral blood lymphocytes of a man immune to *Leishmania major* (LRC-L137). For establishment of the anti-LTL,  $25 \times 10^6$  mononuclear cells were stimulated with the supernatant of 1 mg of a freeze-thawed-sonicated and centrifuged preparation of *L. major* parasites, and 6 days later transferred to GM, with IL-2-containing supernatant from phytohemagglutinin (PHA)-stimulated human mononuclear cells. Anti-LTL was restimulated with 500 mg FTS and  $25 \times 10^6$  autologous irradiated mononuclear cells per  $25 \times 10^6$  anti-LTL cells.

### Fluorescent analysis of membrane markers

Cells from the AMTL, anti-PPD-TL, and anti-LTL were analyzed for their membrane markers by indirect immunofluorescence as described by Gross et al. (1984). The monoclonal antibodies used for the AMTL and the anti-PPD cells were anti-mouse Thy 1.2 (NEN, U.S.A.) and anti-mouse Lyt 2.2 (a generous gift from Dr. J. Goding, Australia). For the anti-LTL cells, Leu-2 and Leu-3 monoclonal antibodies (Beckton-Dickinson) were used.

### Supernatant

Culture supernatant (SUP) of the anti-malaria and anti-PPD lines was collected 5 days after restimulation. GM was used as control for these supernatants. Anti-LTL-SUP was collected 3 days after restimulation and PM + 10% FCS + FTS served as its control.

### Adoptive transfer of the AMTL cells

*Effect on subsequent infection:* AMTL cells, collected 5 days after restimulation with PE, were injected intravenously into naive mice ( $14 \times 10^6$  viable cells/mouse). GM-injected or untreated mice served as controls. Seven days after injection of the cells or GM, mice were infected with  $5 \times 10^4$  or  $1 \times 10^6$  PE.

*Measurement of delayed-type hypersensitivity (DTH) in normal recipients:* The technique was adapted from the procedure described by Lefford (1974). Naive mice were injected intravenously with  $14 \times 10^6$  viable cells from the AMTL that had been collected 5–6 days after restimulation with PE. Control mice were injected with GM. Mice immune to *P. berghei* were used as a positive control group. Seven days after injection of the cells the mice were given a subcutaneous pulse of 0.35  $\mu\text{Ci}/\text{g}$  body weight of [ $^3\text{H}$ ]thymidine (5 Ci/mMol, Nuclear Research Center, Israel), which labels monocyte and macrophage precursors accumulating at the DTH reaction site. Twenty-four hr later  $50 \times 10^6$  PE or sheep red blood cells in 20  $\mu\text{l}$  RPMI-1640 were injected into 1 hind footpad and  $50 \times 10^6$  NE were injected into the second hind footpad. After 48 hr the mice were killed and the footpads were removed. For radiometric analysis, the footpad tissue was dissolved and counted as described (Lefford, 1984). The ratio of radioactivity uptake of the antigen to the control injected foot of each individual mouse was expressed as stimulation index (SI).

### Measurement of chemotactic locomotion of mouse macrophage and human mononuclear cells

Chemotaxis was measured in modified Boyden chambers, as described by Cates et al. (1978). Macrophages were collected from the peritoneal cavity of normal and infected mice. Mononuclear cells from healthy men were separated from 15 ml of blood drawn with Na-citrate on Ficoll-Hypaque (Uppsala, Sweden) by 20 min of centrifugation at 2,000 rpm. The macrophages and mononuclear cells were washed and adjusted to  $3 \times 10^6$  cells/ml Gey's medium. Millipore filters, 5- $\mu\text{m}$ -pore size (Bel-Gar, France), were used. The chemoattractant (200  $\mu\text{l}$ ) was placed in the lower compartment and the cells (200  $\mu\text{l}$ ) in the upper compartment of the chamber. After 2½ hr incubation at 37°C, the filters were removed, stained as described by Cates et al. (1978), placed between 2 drops of immer-

sion oil on a glass slide, and covered with a cover slip. The maximal vertical migration of the cells was determined as the distance between the plane at the origin and the plane formed by the 3 most distant cells in proximity to the chemotactic material. Spontaneous chemotaxis was determined as the migration of cells in the presence of Gey's medium alone. Zymosan A-activated human serum, 1.5% in Gey's medium (from *S. cerevisiae* yeast, Sigma, U.S.A.) was used as a positive control.

#### Partial characterization of the AMTL-SUP

**Temperature effect:** Crude SUP and the control GM were heated to 56 C for 30 min, or were boiled for 10 min.

**Protease treatment:** Crude SUP and the control GM were treated with insoluble protease attached to carboxymethylcellulose (Sigma), 1 unit/2.5 ml SUP or GM. After 2 hr at 37 C in a shaking bath, the insoluble enzyme was removed by centrifugation.

**Determination of the approximate molecular weight:** AMTL-SUP and GM were passed through Sartorius membrane filters (Goettingen, F.R.G., retention limit 13,200) and through Schleicher and Schuell filters (Dassel, F.R.G., retention limit 10,000).

The inside and outside medium obtained was tested for chemotactic activity. Three ml of AMTL-SUP or control GM were also passed through a 60 × 1.5-cm Sephadex G-100 column (Pharmacia, Uppsala, Sweden). After removal of the void volume 45 2-ml fractions were collected. During the separation, aseptic conditions were maintained to the fullest extent possible and the fractions collected were filtered through 0.45-μm filters (Tamar, Jerusalem) to exclude the presence of bacteria. After protein was estimated by absorbance at 280 nm, the column effluent was collected into 5 fractions. Blue dextran ( $2 \times 10^6$  Daltons), hemoglobin (64,000), cytochrome C (12,600), and vitamin B<sub>12</sub> (1,300) were used as molecular weight markers.

## RESULTS

#### Establishment and characterization of the T-cell lines

Long-term proliferative T-cell lines, AMTL, and 2 control lines were established. The lines responded with blast transformation only to the antigen to which they were sensitized (results not shown). The proliferative response to the sensitizing antigen was not lost during the culture period. By immunofluorescence, using monoclonal antibodies, we demonstrated that after 2 wk in culture, the AMTL and the control lines consisted only of T-lymphocytes with helper phenotype. The lines were IL-2 dependent, and grew in the presence of IL-2-containing medium for a prolonged period.

#### Adoptive transfer of the AMTL cells

**Effect on subsequent infection:** Naive mice were infected with *P. berghei* 7 days after intra-

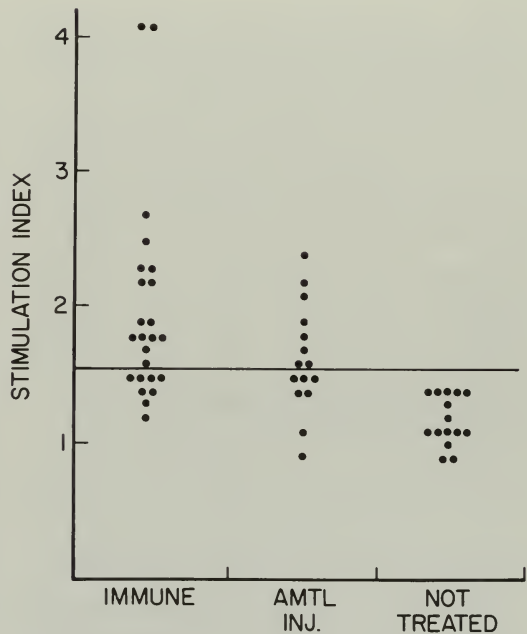


FIGURE 1. Efficacy of antimalaria T-cell (AMTL) cells in the adoptive transfer of delayed hypersensitivity compared to immune mice and normal mice: distribution of responses. The horizontal line represents the average response of normal mice plus 2 SD.

venous injection of AMTL cells. Mice that were not treated before infection served as controls. Three experiments were performed. In each experiment, 36 mice were tested (12 mice per group). In all experiments, the parasitemia in the AMTL-injected mice paralleled the parasitemia observed among the 2 control groups. Parasitemia levels in all the mice reached a peak of 50–70% on day 16 after infection. All mice died between days 16 and 20.

**Delayed-type hypersensitivity (DTH) response in normal recipients:** The radiometric method for the measurement of DTH is based on thymidine uptake by monocytes and macrophages accumulating at the site of reaction. We used this technique in order to evaluate the chemotactic effect AMTL cells might have *in vivo* on monocytes and macrophages. AMTL cells were injected into naive mice and 8 days later DTH was measured. Immune and naive mice were used as controls. The experimental groups included 15–25 mice each. The results of adoptive transfer (Fig. 1) show that the response of the AMTL cell-injected mice was comparable to the response of immune mice and was higher than the response of untreated animals.



TABLE I. Chemotactic locomotion of macrophages from normal and infected mice and healthy human mononuclear cells in the presence of AMTL supernatant, control supernatants, and control media.

	Chemotactic locomotion ( $\mu\text{m}/2.5 \text{ hr} \pm \text{SEM}$ )*		
	Normal mouse macrophages	Infected mouse macrophages	Normal human mononuclear cells
Gey's medium	39.1 $\pm$ 3.5	43.3 $\pm$ 4.4	38.7 $\pm$ 3.2
GM	40.3 $\pm$ 3.1	39.8 $\pm$ 1.9	37.0 $\pm$ 2.1
LTL control	—	—	35.5 $\pm$ 2.4
AMTL-SUP	79.3 $\pm$ 1.9†	46.0 $\pm$ 2.4§	80.0 $\pm$ 3.9‡
PPD-SUP	81.0 $\pm$ 9.1‡	55.6 $\pm$ 2.2‡	57.8 $\pm$ 3.2‡
LTL-SUP	—	—	56.5 $\pm$ 3.6‡
ZAS†	83.6 $\pm$ 2.9‡	51.3 $\pm$ 1.8‡	89.0 $\pm$ 1.0‡

\* Mean values of 6 chemotactic locomotion fields.

† ZAS = nonspecific chemoattractant.

‡ Statistically significant in *t*-test, from control (GM or LTL control) ( $P > 0.005$  at least).

§ Differ significantly from GM ( $P > 0.05$ ).

### Chemotactic activity of the AMTL supernatant

AMTL-SUP, as well as the control anti-PPD-SUP, augmented chemotaxis of macrophages of both normal and infected mice (Table I). However, the locomotion of macrophages from normal mice in the presence of the supernatants and in the presence of the nonspecific chemoattractant ZAS was much greater than the locomotion of macrophages from infected mice. The control medium (GM) did not enhance locomotion of macrophages, as compared to the spontaneous locomotion in the presence of Gey's medium. AMTL-SUP, and the 2 control supernatants (PPD-SUP and LTL-SUP) also enhanced chemotaxis of healthy human mononuclear cells (Table I).

### Biochemical properties of the AMTL-SUP

**Effect of heat and protease treatment:** Heating (56 C for 30 min), boiling, and protease treatment significantly decreased the activity of the

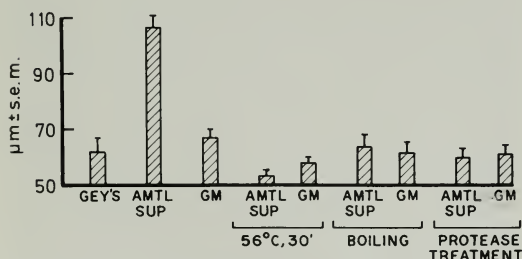


FIGURE 2. Chemotaxis of macrophages from normal mice in the presence of antimalaria T-line supernatant (AMTL-SUP) or growth medium (GM) before and after heat and protease treatment.

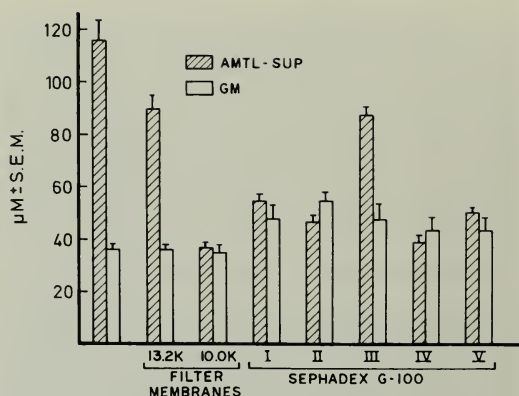


FIGURE 3. Mononuclear cell chemotaxis in the presence of antimalaria T-line supernatant (AMTL-SUP) or control growth medium (GM) before and after exclusion by membrane filters and in the presence of AMTL-SUP fractions eluted from a Sephadex G-100 column. The activity of the fractions was compared to untreated controls, on the far left. Molecular weight of the Sephadex G-100 fractions: I: 64–90 kDa; II: 13–64 kDa; III: 12.6 kDa; IV, V: <12.6 kDa.

AMTL-SUP as chemoattractant (Fig. 2). These results indicate that proteins in the supernatant were involved in the induction of chemotaxis.

**Approximate molecular weight of the active components:** The active AMTL-SUP component that enhanced chemotaxis was excluded by a 13,200- but not by a 10,000-Dalton membrane filter (Fig. 3). After separation on a Sephadex G-100 column, the chemotactic activity of the AMTL-SUP was detected in the fraction with a molecular weight of about 12,600 Daltons (Fig. 3).

### DISCUSSION

T-lymphocytes play an essential role in the immune response to malaria infection. In various experimental systems, adoptive transfer of enriched populations of helper T cells (Lyt 1.2<sup>+</sup> or Lyt 2.2<sup>+</sup>) to mice improved their resistance to malaria (Tanabe et al., 1977; Finley et al., 1983; Brake et al., 1986). In contrast to the work of Brake et al., in the present study the use of a T-cell line injected into naive mice did not confer immunity to infection with *P. berghei*. This finding prompted us to ascertain whether the failure of the AMTL cells to improve the immune response to malaria was due to an intrinsic defect in the helper T-cell line developed, or to malfunction of other components of the immune system interactive with T-cells, such as macrophages.

Defects in macrophage function during malaria have been reported (Greenwood et al., 1971; Weidanz and Rank, 1975; Loose and DiLuzio, 1976; Warren and Weidanz, 1976; Tanabe et al., 1977; Murphy, 1981), but little is known about the effect of malaria on macrophage motility. This was of interest to us because it is known that specific lymphocytes produce lymphokines that act as chemoattractants (Wyler and Gallin, 1977). We therefore evaluated the effect of the AMTL cells or their supernatant on macrophage motility, *in vivo* and *in vitro*. *In vivo*, it was shown that normal mice injected with AMTL cells demonstrated a cutaneous DTH response to PE. This indicates that the AMTL cells, acting through lymphokines, were able to function *in vivo* and to attract normal granulocytes and macrophages to the site of PE injection. *In vitro*, it was found that AMTL-SUP augmented the chemotactic response of macrophages derived from both normal and infected animals. However, the effect on macrophages derived from normal mice was significantly higher. It appears that macrophages from infected mice have a diminished ability to respond to the T-lymphocyte-mediated stimuli.

The chemotaxis induced by the AMTL-SUP was found to be similar to the activity induced by the supernatant of 2 control T-cell lines (anti-*Leishmania* and anti-tuberculin) indicating that the ability of the AMTL cells to produce chemotactic lymphokines was probably intact. Therefore it appears that T-cells sensitized to malaria antigens are able to interact with normal macrophages through lymphokines that stimulate macrophage motility. We suggest that the defects in macrophage activity observed during malaria infection in mice are inherent to the macrophage and are not due to T-lymphocyte malfunction.

#### ACKNOWLEDGMENTS

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## TAENIA TAENIAEFORMIS: CELLULAR RECONSTRUCTION OF ATHYMIC MICE AND ROLE OF L3T4<sup>+</sup> HELPER T LYMPHOCYTES IN THE EARLY INFECTION

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**ABSTRACT:** The role of T helper lymphocytes (L3T4<sup>+</sup>) in the early response to *Taenia taeniaeformis* metacystodes was investigated. Athymic BALB/c-*nu/nu* mice (susceptible) were inoculated intraperitoneally with the following cell populations from congenic BALB/c-*nu/+* mice (resistant): (a) whole spleen single cells, (b) thymus single cell suspensions, or (c) spleen cells pretreated with anti-L3T4 monoclonal antibody before the injection. The mice were given 3 weekly injections of cells and then infected orally with 300 eggs 7 days after the last injection. Cryostat sections of the liver from the infected mice were examined at 6 days postinfection (PI) for parasite viability, the numbers of eosinophils, and L3T4<sup>+</sup> T lymphocytes present within 100  $\mu$ m of the parasite and for the presence of biotin in hepatocytes (involved in biosynthesis of fatty acids) around the parasite. The success of the cellular reconstitution of athymic mice with the lymphoid cells was measured by a T-cell mitogenic assay with concanavalin A (ConA). The cellular reconstitution of athymic mice with a mixture of lymphoid cells from the spleen and thymus of BALB/c-*nu/+* mice resulted in both parasite death and eosinophil infiltration. Reconstitution with mature splenic cells alone resulted in a greater parasite killing and eosinophil infiltration as compared to reconstitution with thymic cells. The better reconstitution with splenic cells was reflected in a greater mitogenic response to ConA. Anti-L3T4 treatment of spleen cells before the inoculation into athymic mice caused a severe depletion of the L3T4<sup>+</sup> cell population that resulted in: (a) elimination of L3T4<sup>+</sup> cells in the parasite area in the liver, (b) elimination of the parasite killing effect from the whole spleen cell population, (c) little or no eosinophil infiltration, and (d) no accumulation of biotin in the parasite-adjacent hepatocytes. By all these parameters, control athymic mice showed no response to the parasite, whereas heterozygous mice and whole spleen cell-reconstituted athymic mice were positive to the indicators of host-parasite interaction. Based on these results, 2 hypotheses are discussed concerning the function of L3T4<sup>+</sup> helper T lymphocytes during the early phase of resistance of BALB/c mice.

It has been demonstrated recently that the initial response of resistant mice to the metacystode form of *Taenia taeniaeformis* occurred within the first 6 days of infection (Letonja et al., 1984). This early phase of resistance was characterized by a heavy eosinophilic infiltration into the liver to the site of the metacystode that appeared between 3 to 4 days postinfection (PI) (Letonja and Hammerberg, 1987b). Parasite death was identifiable as early as 5 days PI in the resistant strains of mice (Letonja and Hammerberg, 1987a). During this period, antibodies against oncosphere antigens were not detected (Davis and Hammerberg, 1988). A possible role for T cells in controlling the immune response to *T. taeniaeformis* is suggested by the work of Mitchell et al. (1977). It has been proposed by Williams (1982) that this T-cell involvement may not simply be due to providing help to B cells.

In this report, cellular reconstitution of athymic (nude) mice that are highly susceptible to *T. taeniaeformis* infections (Mitchell et al., 1977) was used in combination with treatment with a monoclonal antibody (MAb) against the L3T4 lymphocyte surface antigen, to determine the role of T helper cells (L3T4<sup>+</sup>) in the early response period.

### MATERIALS AND METHODS

#### Animals

Female congenic BALB/c-*nu/nu* and BALB/c-*nu/+* mice, 4-6 wk old, were purchased from Taconic Inc. (Germantown, New York). Athymic (*nu/nu*) mice were maintained in filtered-barrier cages, which together with the food and water were autoclaved as a unit. Athymic mice were handled with sterile gloves in a horizontal laminar flow hood. *Taenia taeniaeformis*-infected cats were kept in the laboratory animal facilities of North Carolina State University, School of Veterinary Medicine. All animals were given brand food and water *ad libitum*.

#### Parasite infection

BALB/c-*nu/nu* and BALB/c-*nu/+* mice were dosed per os with 300 eggs of *T. taeniaeformis*. The strain of *T. taeniaeformis* used was derived originally from gravid segments isolated by Mr. C. E. Claggett in the Laboratory of Parasitic Diseases, National Institutes of Health.

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Health, Bethesda, Maryland (Leid and Williams, 1974), and have been maintained in cats in captivity for several years. Eggs were recovered from segments eliminated by cats infected with rat-generated mature strobilocerci. Eggs were washed and stored at 4°C in phosphate-buffered saline (PBS) containing penicillin, streptomycin, and nystatin.

### Cellular reconstitution

Splenocyte and thymocyte single cell suspensions from BALB/c-*nu*/+ mice were prepared in Hanks' balanced salt solution (HBSS). Splenic cells were treated 1 time with 0.16 M  $\text{NH}_4\text{Cl}$  in 0.02 M Trisma (pH 7.2). Cells were washed 3 times with HBSS and resuspended at  $5 \times 10^7$  cells/ml HBSS. Some splenic cell preparations were also treated with a MAb against L3T4 murine T cells (rat IgG2b) obtained from Accurate Chemical and Scientific Corp. (Westbury, New York). One ml of cell suspension was incubated with 200  $\mu\text{g}$  of the partially purified MAb (by 50%  $[(\text{NH}_4)_2\text{SO}_4]$  precipitation) for 15 min at room temperature. The mixture of splenic cells ( $5 \times 10^7$ ) plus monoclonal antibody (200  $\mu\text{g}$  protein) was then injected intraperitoneally (i.p.) into the athymic mice. Athymic mice were given 3 weekly i.p. injections of either (a)  $5 \times 10^7$  untreated cells from a pool of 11 *nu*/+ mice, (b) MAb-treated cells from a pool of 21 *nu*/+ mice, or (c) 1% bovine serum albumin-PBS before infection with 300 eggs of *T. taeniaeformis* 7 days after the last injection. Four athymic mice and 4 control mice were used in each type of cellular reconstitution treatment.

### Liver processing

Six days PI mice were killed by cervical dislocation and livers removed. Livers were processed for cryostat sectioning as described previously (Letonja and Hammerberg, 1987a). Six- $\mu\text{m}$  sections were stained for eosinophils by a modified Congo red method (Tarpley et al., 1984) and counterstained with Harris' hematoxylin (Letonja and Hammerberg, 1987b). Slides were made from 3 different pieces of liver per mouse and parasite viability and eosinophil infiltration within 100  $\mu\text{m}$  of the parasite were determined as described by Letonja and Hammerberg (1987a, 1987b). Immunohistochemistry on the liver section was performed with rat MAb against L3T4 (IgG2b) or the O-side chain of *Brucella* (IgG2b) as a negative control. Hybridomas producing the rat MAb against L3T4 (GK1.5) were obtained from the American Type Culture Collection, and the O-side chain of *Brucella* (2-28-1) was kindly supplied by Dr. G. G. Schurig (Schurig et al., 1984). Supernatants from both hybridomas were collected in RPMI-1640 medium with 10% Nu-serum (Collaborative Research, Inc., Bedford, Massachusetts). The rat immunoglobulins were isolated by affinity chromatography using an anti-rat IgG (Cappel Lab., Cochranville, Pennsylvania) coupled to cyanogen bromide-activated Sepharose (Sigma, St. Louis, Missouri). The immunohistochemistry method and the detection of accumulation of biotin in the hepatocytes adjacent to the parasite were performed by the binding of the avidin-biotin complex (ABC) as described previously (Letonja and Hammerberg, 1987a). Stained mononuclear cells with 100  $\mu\text{m}$  of the parasite were counted. An average of

12 metacystode sites per mouse was examined for the enumeration of eosinophils and the MAb-labeled cells.

### Mitogenic assay

Cell suspensions were prepared from spleens of 6-days-PI mice. These splenic cells were treated 1 time with 0.16 M  $\text{NH}_4\text{Cl}$  in 0.02 M Trisma (pH 7.2), washed 3 times with HBSS, and resuspended at  $5 \times 10^6$  cells/ml of RPMI-1640 with 10% horse serum (HS) and supplemented with  $5 \times 10^{-5}$  M 2-mercaptoethanol, sodium penicillin G (500 U/ml), streptomycin (50  $\mu\text{g}$ /ml), 5 mM HEPES, and 2 mM L-glutamine; 100  $\mu\text{l}$  of cells were added to 100  $\mu\text{l}$  of medium or concanavalin A (ConA) (0.05 mg/ml) in flat-bottom 96-well tissue culture plates and incubated for 48 hr at 37°C, 5%  $\text{CO}_2$  in air. Cells were then incubated for another 18 hr in the presence of 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine (New England Nuclear, Boston, Massachusetts). After freezing and thawing, cells were harvested on a multiple automated sample harvester (MASH) and counted in Ecoscint (National Diagnostics, Manville, New Jersey) in a Beckman liquid scintillation system. Results are given as the percentage of the mitogenic stimulation obtained by the *nu*/+ splenic cells.

### Flow cytometry

Splenic cells for use in flow cytometry were resuspended at  $4 \times 10^7$  cells/ml of PBS-5% HS. One hundred  $\mu\text{l}$  of  $2 \times 10^7$  splenic cells were mixed with the MAb (75  $\mu\text{g}$ /ml) for 45 min on ice. After 2 washes, 100  $\mu\text{l}$  of goat anti-rat IgG (heavy and light chain) fluorescein isothiocyanate (FITC)-conjugated (mouse absorbed) (Rupp and Bowman, Mt. Laurel, New Jersey) at a concentration of 70  $\mu\text{g}$ /ml was added directly to the cell pellet. After incubating for 45 min on ice and washing 2 times with PBS plus 5% HS, cells were resuspended in 0.5 ml of PBS-5% HS. Cells were filtered through a nylon mesh (35  $\mu\text{m}$ ) before flow cytometry on a Coulter EPICS V cell sorter. Residual erythrocytes were gated out and 10,000 cells per sample were analyzed. The number of fluorescent signals above the control, 2-28-1 MAb rat immunoglobulin, was determined by subtracting the control from the MAb reactive to L3T4 fluorescent events. The remaining number of cells was expressed as a percentage of the total number of cells counted.

### In vivo inflammatory response

BALB/c-*nu*/*nu* and BALB/c-*nu*/+ mice were injected with 1.5 ml of thioglycollate medium i.p. (Difco Laboratories, Detroit, Michigan). After 48 hr, the peritoneal cavity was washed with 5 ml HBSS. Cells were washed 2 times and resuspended in HBSS and counted. The number of eosinophils was determined by the method of Discombe (1946). Macrophages were stained by an alpha-naphthyl acetate method for esterase activity (Davis, 1959).

## RESULTS

### Effect of cellular reconstitution upon the early phase of resistance

Athymic mice, in contrast to the heterozygotes, were completely susceptible to the early stage (pre-6 days PI) of *T. taeniaeformis* infection

TABLE I. Cellular reconstitution of athymic mice (nu/nu) with whole splenic or thymic cell populations from congenic heterozygotes (nu/+).

Experiment	Mouse infected	Cell population (reconstitution)	Parasite viability (mean $\pm$ SD)	Eosinophil infiltration (mean $\pm$ SD)	% Mitogenic response of heterozygote
A	nu/+	None	52 $\pm$ 22	277 $\pm$ 108	
	nu/nu	None	100	0	1
	nu/nu	Thymus/spleen	77 $\pm$ 3	42 $\pm$ 47	69
B	nu/+	None	19 $\pm$ 12	103 $\pm$ 40	
	nu/nu	None	100	0	2
	nu/nu	Thymus	75 $\pm$ 14	19 $\pm$ 21	46
	nu/nu	Spleen	48 $\pm$ 8	67 $\pm$ 46	53

(Table I). This total susceptibility was associated with a complete lack of eosinophil infiltration. In experiment A, cellular reconstitution of athymic mice with a mixture of lymphoid cells from the spleen and thymus of heterozygous mice, according to the temporal procedure of Mitchell et al. (1977), resulted in both parasite death and eosinophil infiltration. In experiment B when separate groups of athymic mice received equivalent numbers of either thymic or splenic cells, reconstitution with the more mature splenic cell population resulted in greater parasite killing and eosinophil infiltration. The more successful cellular reconstitution with splenic cells was reflected in the greater mitogenic response of splenic cells to ConA from the athymic mice given heterozygous splenic cells as compared to thymic cells (Table I).

#### Result of treatment with anti-L3T4 monoclonal antibody

A preliminary experiment where heterozygous splenic cells were treated with 100  $\mu$ g (protein) anti-L3T4 MAb resulted in elimination of any parasite killing and eosinophil infiltration in athymic mice that was induced by cellular reconstitution with heterozygous splenic cells. The anti-L3T4 treatment was accompanied by a reduction of the percent mitogenic response of the heterozygous mitogenic response from 31% (untreated splenic cells) to 14% (anti-L3T4-treated splenic cells), indicating a reduction in functional

T cells (data not shown). This experiment was repeated with the amount of monoclonal antibody increased to 200  $\mu$ g (protein) per mouse treatment. The presence of L3T4<sup>+</sup> cells in the spleen and liver of cellular-reconstituted athymic mice at 6 days PI was examined by flow cytometry and immunohistochemistry, respectively (Table II). The number of L3T4<sup>+</sup> cells in the spleens of athymic mice reconstituted with untreated heterozygous splenic cells was half of that found in the heterozygous spleen. The anti-L3T4 treatment resulted in no detectable L3T4<sup>+</sup> cells in the spleen of cellular-reconstituted athymic mice. Anti-L3T4 treatment also eliminated the L3T4<sup>+</sup> cells around the parasite in the liver. The lack of L3T4<sup>+</sup> cells around the parasite in the liver of heterozygotes was probably due to the high parasite mortality (82% dead) and a resulting exodus of such cells from the site of infection. Both of the above assays would indicate that the anti-L3T4 treatment severely depleted the L3T4<sup>+</sup> cell population, even though the reduction in the mitogenic response (Table III) was not as great as the preliminary experiment. This mitogenic response can be attributed to the remaining Lyt-2<sup>+</sup> cells in the spleen (Kern et al., 1987).

The effect of anti-L3T4 monoclonal treatment upon the host-parasite interaction in cellular-reconstituted athymic mice was determined by examining the livers from these mice 6 days PI by 3 parameters: parasite death (Table III), eosinophil infiltration (Table III), and hepatocytic

TABLE II. Number of L3T4<sup>+</sup> mononuclear cells in the spleen and within 100  $\mu$ m of parasite in the liver of 6-days-PI cellular-reconstituted MAb-treated athymic mice.

Mouse infected	Cells used for reconstitution	MAb treatment	% L3T4 <sup>+</sup> splenic cells (mean $\pm$ SD)	Number of L3T4 <sup>+</sup> cells per parasite site (mean $\pm$ SD)
nu/+	None	None	15 $\pm$ 3	2 $\pm$ 2
nu/nu	None	None	0	0
nu/nu	Splenic cells	None	7 $\pm$ 4	16 $\pm$ 10
nu/nu	Splenic cells	Anti-L3T4	0	1 $\pm$ 1



TABLE III. Effect of the treatment with anti-L3T4 MAb upon cellular reconstitution in athymic mice.

Mouse infected	Cells used for reconstitution	MAb treatment	Parasite viability (mean ± SD)	Eosinophil infiltration (mean ± SD)	% Mitogenic response of heterozygote
nu/+	None	None	18 ± 6	80 ± 31	—
nu/nu	None	None	100	0	3
nu/nu	Spleen	None	65 ± 6	74 ± 48	51
nu/nu	Spleen	Anti-L3T4	100	2 ± 2	40

biotin accumulation (Table IV). By all 3 measurements, athymic mice showed no response to the parasite. In these mice there was no parasite mortality, eosinophil infiltration, or detectable binding of the avidin-biotin complex to hepatocytes adjacent to the parasite (Fig. 1). In contrast, heterozygous mice were positive for all 3 indicators of host-parasite interaction: parasite killing, eosinophil infiltration, and biotin accumulation (Fig. 2). Athymic mice given untreated heterozygous splenic cells had a definite but reduced parasite killing, a definitive but more variable eosinophil infiltration, and hepatocyte biotin accumulation comparable to the heterozygous mice (Figs. 3, 4). However, the anti-L3T4 MAb treatment resulted in an elimination of any parasite killing, little or no eosinophil infiltration, and no accumulation of biotin in the hepatocytes adjacent to parasites. Unlike the unreconstituted athymic mice that had few inflammatory cells, noneosinophilic polymorphonuclear leukocytes were observed in the anti-L3T4 MAb-treated cellular-reconstituted athymic mice (Fig. 5).

**In vivo chemotactic ability of athymic eosinophils**

Because parasite mortality appeared to be associated with eosinophil infiltration, the ability of eosinophils of athymic mice to respond *in vivo* to a nonspecific chemotactic signal, thioglycolate medium, was examined (Table V). The athymic mice had a better inflammatory response, in terms of the overall cellular response, compared to heterozygous mice. The relative response of eosinophils of athymic mice was comparable to that of the heterozygotes. Inflammatory cells, in general, and eosinophils, in particular, from athymic mice appeared capable of responding to an *in vivo* chemotactic signal.

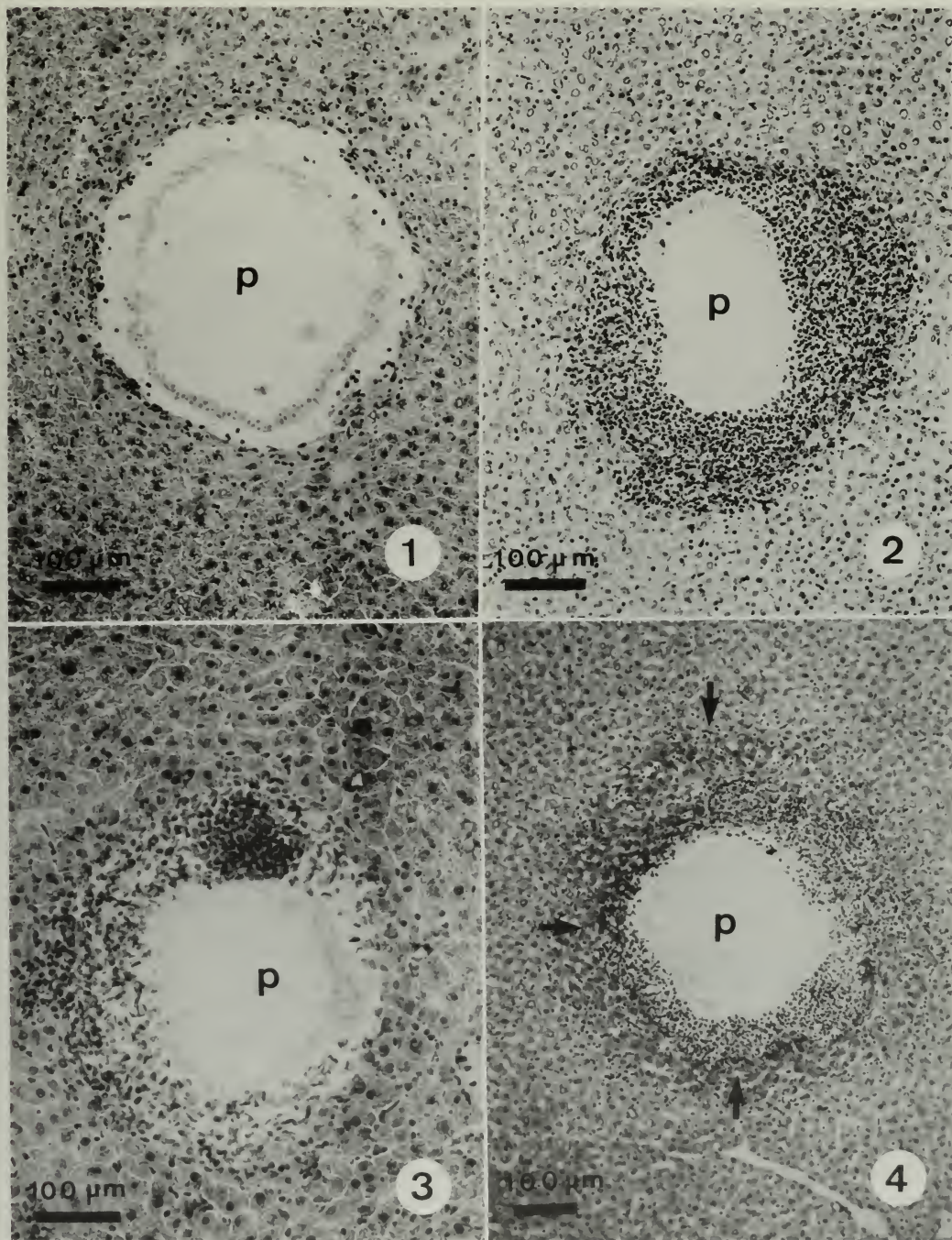
**DISCUSSION**

Cellular reconstitution of athymic mice with lymphoid cells from either the thymus or spleen of congenic heterozygous mice (nu/+) provided protection in the early infection phase (pre-6 days

PI) of the metacystode form of *T. taeniaeformis*. This protection appeared to be dependent upon 2 factors: (a) the appearance of eosinophils near the parasite and (b) cellular reconstitution with a mature lymphoid cell population. Even though heterozygous thymic cells by themselves provided some degree of protection in athymic mice, cellular reconstitution with the more mature heterozygous splenic cells resulted in higher parasite mortality and eosinophil infiltration. Complete protection was never obtained, which was probably due to incomplete cellular reconstitution with functional T cells, because the mitogenic response of splenic cells from cellular reconstituted athymic mice was always lower than the mitogenic response of heterozygous splenic cells. Treatment of heterozygous splenic cells with a MAb against the L3T4 molecule, a cell-surface antigen found on T helper cells (Dialynas et al., 1983), resulted in total elimination of any early-phase protection and accompanying eosinophil infiltration. In addition, preliminary results (data not shown) of treatment of spleen cells with a MAb of the same rat subclass as the anti-L3T4, but against Lyt-2, a cell-surface molecule found on T suppressor/cytotoxic cells (Ledbetter and Herzenberg, 1979), had no effect on parasite mortality but did result in increased eosinophil infiltration into the 6-days-PI parasite site of cellular-reconstituted athymic mice. It would appear that because most of the parasite deaths occurred by 6 days PI, that the T helper (L3T4+) cell involvement in the host's response occurred within this time period. Mitchell et al. (1977)

TABLE IV. Effect of the treatment with anti-L3T4 upon accumulation of biotin in hepatocytes surrounding developing metacystodes.

Mouse infected	Cells used for reconstitution	MAb treatment	Binding of avidin-biotin complex
nu/+	None	None	+
nu/nu	None	None	—
nu/nu	Splenic	None	+
nu/nu	Splenic	Anti-L3T4	—



FIGURES 1–4. Six-days-PI metacystode (p) of *T. taeniaeformis* in the liver of BALB/c-*nu/nu* and BALB/c-*nu/+* mice. 1. Control athymic mice without inflammatory cell infiltration (Congo red). 2. Heterozygous mice with dense inflammatory cell infiltration (avidin-biotin complex stain). 3. Athymic mice given untreated heterozygous splenic cells showing heavy inflammatory cell infiltration (Congo red). 4. Athymic mice given untreated heterozygous splenic cells showing biotin accumulation in hepatocytes (avidin-biotin complex stain, arrows). Bar = 100  $\mu$ m.



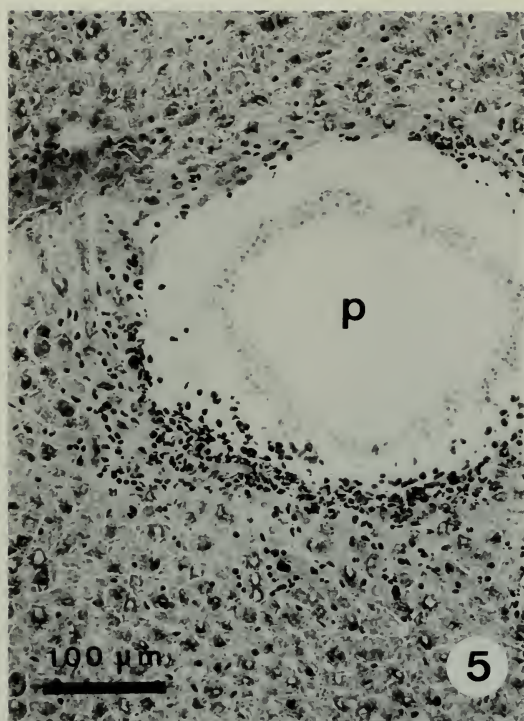


FIGURE 5. Liver section from athymic mouse pretreated with anti-L3T4 MAb with noneosinophil polymorphonuclear leukocytes around a 6-days-PI parasite (p) Congo red stain. Bar = 100  $\mu$ m.

had previously demonstrated a probable requirement for T cells, using an observation period of 3 wk, in the response of athymic mice to *T. taeniaeformis*. In addition, these workers found that giving athymic mice lymphoid cells 10 days PI had no influence upon the survival of the larvae, thus supporting our suggestion that the T helper (L3T4<sup>+</sup>) cell involvement occurs within the first 6 days of infection.

The requirement for T helper (L3T4<sup>+</sup>) cells in the early phase of resistance was associated with eosinophil infiltration around the parasite in the liver. T helper cells may influence the eosinophil migration response by 2 mechanisms: (a) production of a chemotactic signal such as eosinophil stimulation promotor (Greene and Colley, 1976) at the site of the parasite or (b) production of a systemic eosinophil-stimulating factor that would increase the ability of eosinophils to respond to a chemotactic signal (Silberstein and David, 1987). The latter mechanism would be important if eosinophils in athymic mice have a decreased ability to respond to an *in vivo* chemotactic signal. However, this is not the case, as

TABLE V. Peritoneal inflammatory response to intraperitoneal injection of thioglycollate in athymic and heterozygous congenic mice.

Mouse infected	Peritoneal exudate cells (10 <sup>6</sup> ) (mean $\pm$ SD)	% Eosinophils (mean $\pm$ SD)	% Macrophages (mean $\pm$ SD)
nu/+	6.3 $\pm$ 3.8	7.6 $\pm$ 1.9	52 $\pm$ 14.7
nu/nu	17.7 $\pm$ 5.5	7.3 $\pm$ 3.1	79 $\pm$ 4.5

athymic mice inflammatory cells, including eosinophils, were capable of responding to the non-specific chemotactic signal of i.p. thioglycollate injection. If eosinophils in athymic mice are capable of responding to a chemotactic signal, then the lack of eosinophil responsiveness in the *T. taeniaeformis* infection may be due to the lack of production of a chemotactic signal at the parasite site. Two possibilities are suggested: (a) T helper (L3T4<sup>+</sup>) cells are present at the parasite site and are producing a chemotactic factor or (b) a chemotactic signal is produced as a result of some host-parasite interaction. It has been demonstrated previously that T cells were not present in large numbers at any time during the first 6 days of infection (Letonja and Hammerberg, 1987a). Only at 5 days PI were T cells present in numbers that on average exceeded 10 cells per parasite section in the resistant BALB/c strain of mice. However, the influx of eosinophils began between 3 and 4 days PI (Letonja and Hammerberg, 1987b), which suggested that T cells are not directly responsible for producing a local eosinophil chemotactic signal. Davis and Hammerberg (1988) have demonstrated a partial involvement of complement activation in eosinophil infiltration. However, production of an eosinophil chemotactic factor via complement activation probably is not required for eosinophil infiltration because the complement chemotactic peptide C5a is not generated in C5-deficient mice, B10.D2oSn, yet these mice have normal eosinophil infiltration and are resistant to *T. taeniaeformis* infection (Davis and Hammerberg, 1988).

The association of biotin accumulation in hepatocytes adjacent to the parasite with the presence of L3T4<sup>+</sup> T cells in cellular-reconstituted athymic mice suggests a more interesting and speculative possibility: T helper (L3T4<sup>+</sup>) cells mediate an interaction between hepatocytes and the parasite that alters the hepatocytes' lipid metabolism, which may result in the production of a chemotactic factor. Increased biotin concen-

tration in hepatocytes adjacent to the developing metacestode cannot be due simply to the physical presence of the metacestode in the liver of athymic mice because hepatocytes of infected athymic mice did not react with the avidin-biotin complex. Nor is the biotin accumulation likely to be a result of the influx of inflammatory cells, because cellular-reconstituted athymic mice treated with the anti-L3T4 MAb showed infiltrating neutrophils but not eosinophils, yet no binding of the avidin-biotin complex to the hepatocytes was observed. The implication is that hepatocytes that had developed in an environment containing T helper (L3T4<sup>+</sup>) cells are capable of interacting with the parasite. This interaction in mice of the BALB/c background leads to changes in lipid metabolism as indicated by the observed increase in levels of biotin, which is involved in the biosynthesis of fatty acids, and the previously reported increased Sudan black staining of hepatocytes adjacent to the parasite as early as 2 days PI (Letonja and Hammerberg, 1987b).

We propose 2 possible functions for L3T4<sup>+</sup> T cells in the early phase of resistance of BALB/c mice to *T. taeniaeformis*. The more conservative hypothesis suggests that L3T4<sup>+</sup> T cells are activated by parasite antigenic products that result in the production of cytokines involved in the maturation and activation of eosinophils leading to increased eosinophil migration toward a chemotactic signal (Silberstein and David, 1987). Although eosinophils from BALB/c athymic mice are capable of responding to a massive chemotactic signal such as thioglycolate, a more subtle signal produced by the parasite itself (Camp and Leid, 1982) or from activation of the complement cascade (Davis and Hammerberg, 1988) may require eosinophils in a more activated state.

However, if eosinophils from athymic mice are capable of responding to the chemotactic signal released into the parasite's environment, the absence of infiltrating eosinophils in athymic or anti-L3T4-treated cellular-reconstituted athymic mice must be due to the lack of production of the chemotactic signal. This suggests a more speculative hypothesis for the function of L3T4<sup>+</sup> cells as producers of cytokines that influence hepatocyte development such that these cells can be stimulated by the invading metacestode to produce an eosinophil chemotactic factor of lipidic nature. Such a hypothesis is supported by the observation of thymus involvement in the development of hepatocytes (Giuli et al., 1980) and the demonstration of a lipid product of ro-

dent hepatocytes capable of chemotactic activity for polymorphonuclear cells (Pérez et al., 1984). This parasite-liver-T cell interaction may not be restricted to *Taenia* infections. It has been demonstrated that the inflammatory response to infection with *Fasciola hepatica*, including tissue eosinophilia and bile duct hyperplasia, is dependent on the thymus (Eriksen, 1980). Also, cytokines produced by *Schistosoma mansoni* antigen-reactive T-cell clones can directly stimulate fibroblast proliferation (Lammie et al., 1986). Even though the murine schistosomiasis system represents a chronic, granulomatous hepatic infection, it does provide evidence for the hypothesis that T helper cells influence the nonlymphoid cells of the liver and host's response to *T. taeniaeformis* infection.

The 2 proposed functions are not mutually exclusive, but may reflect temporal differences in the function of L3T4<sup>+</sup> cells; the first function would be the immediate response to the presence of parasite products, whereas the second function would be a long-term developmental effect on hepatocyte differentiation. The presence of L3T4<sup>+</sup> cells capable of responding to parasite products with a liver that developed in the presence of L3T4<sup>+</sup> cells can be determined by *in vivo* anti-L3T4 treatment of normal BALB/c mice. The ability of hepatocytes from athymic mice to produce chemotactic factors in response to the metacestode can be investigated *in vitro* using cultured hepatocytes from normal, athymic, cellular-reconstituted athymic, and anti-L3T4-treated cellular-reconstituted athymic BALB/c mice.

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## ANALYSIS OF VARIABLES ASSOCIATED WITH PROMOTION OF RESISTANCE AND ITS ABROGATION IN T CELL-RECONSTITUTED NUDE MICE INFECTED WITH *LEISHMANIA MAJOR*

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**ABSTRACT:** Upon intradermal challenge with the protozoan parasite *Leishmania major*, some mouse strains develop chronic cutaneous lesions, whereas other mouse strains show a resolving pattern of disease. The importance of T cell-dependent immunity in resistance to cutaneous leishmaniasis is substantiated by the susceptibility to infection of athymic nude mice of both resistant and susceptible strains. Small numbers of T lymphocytes from uninfected euthymic mice promote resistance in nude mice but T cells from chronically infected mice can impair this protective effect. In the present study we used an adoptive transfer system in which nude mice were reconstituted with T cells from normal or chronically infected mice in order to further investigate protection against disease or disease promotion. The results supported the following conclusions: (a) the host-protective activity of T cells from uninfected mice is highly effective even in long-term chronically infected nude mice, (b) T cell-mediated exacerbation of cutaneous disease does not involve enhancement of lesion development and is thus unlikely to be based on an accelerated proliferation of parasites in the lesion, (c) disease-promoting cells are not only found in genetically susceptible mice but can also be induced in genetically resistant mice, and (d) lymphoid organs of genetically susceptible mice chronically infected with *L. major* contain resistance-promoting cells in addition to disease-promoting cells. The data, together with those of others, continue to support the notion that recruitment with expansion and/or activation of different T cell subsets underlies genetically based resistance and susceptibility of mice to *L. major*.

In a murine model of cutaneous leishmaniasis, reconstituted athymic nude mice have been used to establish that L3T4<sup>+</sup> T cells are involved in promotion of resistance and promotion of disease (Mitchell et al., 1987; Moll et al., 1988). In this disease (Old World or zoonotic cutaneous leishmaniasis), infection is initiated by promastigotes of *Leishmania major* that invade macrophages, inside which they transform to amastigotes and multiply. The bulk of evidence indicates that the infected macrophage is the target of aggressive immune attack in resistant individuals (Louis and Milon, 1987).

Euthymic mice of BALB/c genotype differ from CBA/H and C57BL/6 mice in being nonhealers after cutaneous injection of *L. major* promastigotes. In our laboratory, using the *L. major* parasite line V121, both CBA/H and C57BL/6 mice are resistant to infection and usually develop only small lesions that heal rapidly within 1-2 mo. Cell transfer experiments have shown small numbers of lymphoid cells from syngeneic euthymic mice ( $10^5$ - $10^6$ ) to be sufficient for establishing total resistance to *L. major* infection in

otherwise susceptible nude mice of BALB/c, CBA/H, and C57BL/6 genotypes (Mitchell et al., 1980). Compatibility at the I-A locus of the MHC is probably necessary for reconstitutive activities of T lymphocytes (Mitchell, 1983). T cells from chronically infected BALB/c mice can abrogate the protective effect of T cells from uninfected mice in BALB/c nude recipients and thus exhibit disease-promoting activity (Mitchell et al., 1981). There is a close similarity between results obtained in the reconstituted nude mouse and the reconstituted sublethally irradiated mouse model used by the Wellcome group (Howard et al., 1980; Howard, 1986; Liew, 1986). Moreover, injection of anti-L3T4 antibodies promotes resistance in BALB/c mice (Titus et al., 1985; Sadtick et al., 1987), a situation reminiscent of the minimally reconstituted BALB/c nude mouse in that only small numbers of L3T4<sup>+</sup> cells lead to resistance. Most of the data are compatible with the notion that resistance-promoting T cells are of high frequency in uninfected euthymic mice relative to disease-promoting T cells but that this ratio is changed in the lymphoid organs of chronically infected BALB/c mice. The 2 functionally different T cell types have the same L3T4<sup>+</sup> Ly-2<sup>-</sup> phenotype and to date cannot be distinguished on the basis of expression of additional surface markers. They may therefore not belong to separate lineages but may rather differ in antigen

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specificity because a glycolipid of *L. major* can promote resistance and a carbohydrate derivative of this antigen can promote disease (Handman and Mitchell, 1985, 1987; Mitchell and Handman, 1986). Recent data from Locksley et al. (1987) strongly suggest that induction of T helper cells of type 1 relative to T helper cells of type 2 (see Mosmann and Coffman, 1987) may underly resistance and susceptibility in mouse strains. The possibility exists that disease-promoting and resistance-promoting T cells have different specificities and produce different mediators.

The mode of action of disease-promoting T cells remains poorly defined. It is generally believed that resistance-promoting L3T4<sup>+</sup> T cells operate by elaboration of lymphokines including interferon- $\gamma$  (Nacy et al., 1983; Titus et al., 1984), or through cell-cell contact (Sypek and Wyler, 1985), to promote activation of the infected macrophage with attendant destruction of the intracellular amastigotes. In the absence of definitive information on how T cells promote lesion persistence, we have continued to explore variables in the basic reconstituted nude mouse system in the expectation that more data will either enhance or reduce the validity of several current hypotheses (Louis and Milon, 1987).

## MATERIALS AND METHODS

### Mice

Female mice of the inbred strains BALB/c, C57BL/6, and CBA/H were used at an age of 7–12 wk. Female athymic BALB/c nu/nu, C57BL/6 nu/nu, and CBA/H nu/nu (nude) mice were 6–8 wk of age at the commencement of experiments. All mice were bred under specific pathogen-free conditions at the Hall Institute but, during experimentation, were maintained under conventional conditions in a small isolation facility.

### Parasites and assessment of lesions

The cloned virulent *L. major* parasite line V121 was produced from the human isolate LRC-L137 (Handman et al., 1983) and maintained by passage in BALB/c mice. Promastigotes were grown *in vitro* in blood/agar cultures (Handman et al., 1979). Stationary-phase promastigotes were washed and were injected in a volume of 50  $\mu$ l intradermally on the dorsum of the mouse close to the base of the tail. Lesion scores were determined at regular intervals according to the following system: 0 = no lesion or healed scar; 1 = small swelling (up to 5 mm in average diameter); 2 = large swelling (more than 5 mm in average diameter) or open lesion of less than 5 mm in average diameter; 3 = open lesion of 5–10 mm in diameter; 4 = open lesion greater than 10 mm in diameter and/or obvious metastases. Data are expressed as the arithmetic mean of the lesion scores for the groups of 4–6 mice and the number of mice

with lesions relative to the total number of mice per group is given for several time points.

### Preparation of cell suspensions

Single cell suspensions were prepared either from normal mouse spleens or from spleens or lymph nodes of mice that had been infected with *L. major* parasites. Cells from chronically infected BALB/c mice were obtained 70–100 days after parasite challenge when the donor mice had lesion scores of 3 and 4, cells from infected C57BL/6 mice were obtained either from currently diseased donors with an unusually high lesion score of 3 or from recovered donors with healed lesions at 68 days of infection. Unseparated or nylon wool-purified T cells (Julius et al., 1973) were injected intraperitoneally in a volume of 0.2 ml and the cell numbers used are given in the legends for individual experiments.

## RESULTS

### Will disease-promoting cells enhance parasite proliferation and rate of lesion development?

T cells from chronically infected mice can impair the host-protective effect of T cells from normal mice and restore permissiveness to *L. major* infection in nude mouse recipients (Mitchell et al., 1981). One possibility to account for this disease-promoting activity of T lymphocytes is that such cells, by unknown mechanisms, promote proliferation of amastigotes, which combined with recruitment of macrophages with inefficient killing capacity (Mirkovich et al., 1986) enhance the spread of the lesion. In order to test this possibility, BALB/c nude mice were injected with  $2 \times 10^7$  nylon wool-purified T cells from chronically infected BALB/c mice and were challenged with either  $10^2$ ,  $10^4$ , or  $10^6$  *L. major* promastigotes,  $10^6$  being the usual number of parasites used for challenge in this laboratory. Control groups of mice were infected with the same numbers of parasites but did not receive any lymphocyte inoculum. It can be seen in Figure 1 that the rate of lesion development is dependent on the number of parasites used for infection and is not increased by injection of T cells. Upon infection with low numbers of promastigotes ( $10^2$  or  $10^4$ ), the presence of T cells with disease-promoting capacity does not give rise to early appearance and rapid expansion of lesions.

### Can resistance-promoting cells induce recovery of chronically infected nude mice?

T lymphocytes from uninfected euthymic mice have the capacity to induce resistance to cutaneous disease in nude mice when administered concomitantly with the parasite challenge. It is

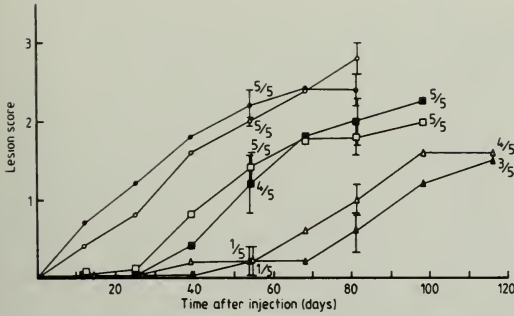


FIGURE 1. Effect of T cells from chronically infected mice on the course of lesion development. On day 0, 5 BALB/c nude mice per group were injected intradermally with  $10^2$  ( $\Delta$ ,  $\blacktriangle$ ),  $10^4$  ( $\square$ ,  $\blacksquare$ ), or  $10^6$  ( $\circ$ ,  $\bullet$ ) promastigotes of *L. major* alone (open symbols) or in addition to  $2 \times 10^7$  T cells from BALB/c mice chronically infected with *L. major* (closed symbols) that were given intraperitoneally. As in succeeding figures, arithmetic means of the cutaneous lesion scores are indicated and the number of mice with lesions relative to the total number of mice per group as well as standard errors are given for certain time points.

conceivable that disease in nude mice may progress to the point that the injection of a limited number of T cells is unable to reverse the course of disease and to induce recovery. CBA/H nude mice were therefore injected intradermally with  $2 \times 10^6$  *L. major* promastigotes and individual groups were reconstituted on days 0, 14, 28, 42, and 56 with  $2 \times 10^7$  nylon wool-purified T cells from euthymic CBA/H mice. It can be seen in Figure 2A that T cells are able to reverse the development of disease in CBA/H nude recipients and induce complete healing of lesions when injected at any time during the course of infection. Even in CBA/H nude mice with lesions greater than 5 mm in diameter, an injection of T cells is fully protective. Similar results were obtained in an equivalent experiment using infected nude mice of BALB/c genotype (Fig. 2B). BALB/c nude mice that received syngeneic T cells up to 42 days after challenge with *L. major* promastigotes were resistant to disease although lesions did not resolve completely in some of the recipients. Only when given at a very late stage of chronic disease (56 days after challenge with parasites) were T cells unable to prevent the fatal effects of infection in this mouse strain. Thus, a firmly established infection, with large numbers of infected macrophages present, can be eliminated by an inoculum of T cells both in genetically resistant and in genetically susceptible mouse strains.

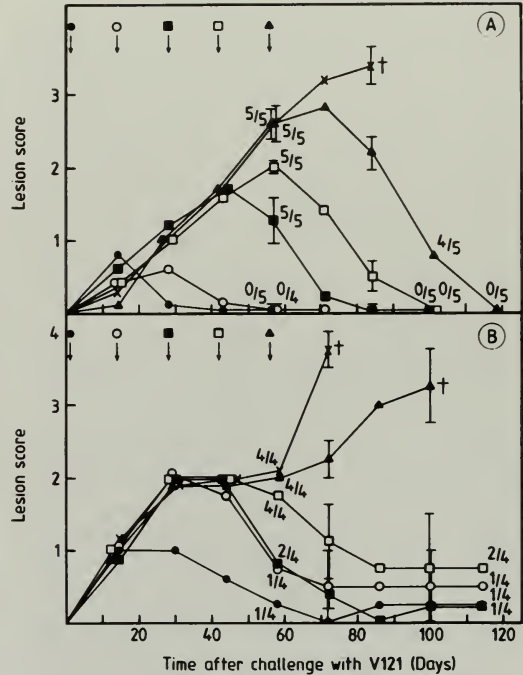


FIGURE 2. Reconstitution of infected nude mice with normal mouse T cells at various time points. On day 0, CBA/H nude mice (A) or BALB/c nude mice (B) were injected intradermally with  $2 \times 10^6$  promastigotes of *L. major*. Individual groups of 4 or 5 nude mouse recipients were injected intraperitoneally with  $2 \times 10^7$  syngeneic T cells from uninfected euthymic mice on days 0 ( $\bullet$ ), 14 ( $\circ$ ), 28 ( $\blacksquare$ ), 42 ( $\square$ ), and 56 ( $\blacktriangle$ ) or were not given any lymphocytes ( $\times$ ). The cross symbols ( $\times$ ) indicate deaths caused by metastases and visceralization of disease.

#### Are resistance-promoting cells present in chronically infected mice?

We have reexamined the question of whether the presence of resistance-promoting cells can be uncovered in a population of lymphoid cells from chronically infected BALB/c mice (Liew et al., 1982; Mitchell, 1983) using a cell titration approach. Upon challenge of BALB/c nude mice with  $10^6$  *L. major* promastigotes and reconstitution with numbers of cells ranging from  $10^2$  to  $10^6$  and  $10^5$  to  $10^8$  in 2 different experiments, it was found that resistance-promoting cells can indeed be detected in chronically infected BALB/c mice (Fig. 3). Nude mice given  $10^4$  or  $10^5$  cells in one experiment and  $10^5$  cells in another were resistant to *L. major* infection, whereas recipients of less than  $10^4$  or more than  $10^5$  cells were susceptible. Thus, it is possible to titrate out disease-promoting cells and to uncover the activi-



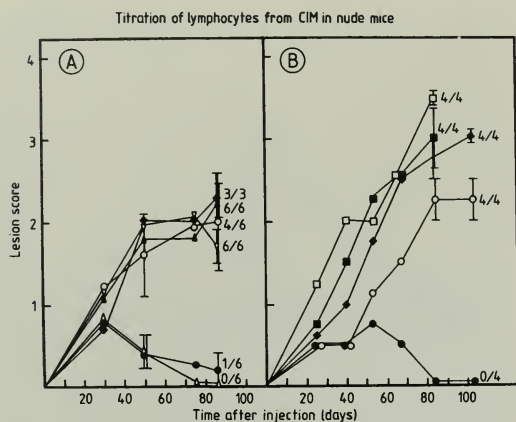


FIGURE 3. Titration of lymphocytes from chronically infected mice in nude mouse recipients. A. Groups of 3 or 6 BALB/c nude mice were injected with  $10^2$  ( $\nabla$ ),  $10^3$  ( $\blacktriangle$ ),  $10^4$  ( $\triangle$ ),  $10^5$  ( $\bullet$ ), or  $10^6$  ( $\circ$ ) pectoral and mesenteric lymph node cells from chronically infected BALB/c mice and were challenged with  $10^6$  *L. major* promastigotes on days 1 and 14. B. Groups of 4 BALB/c nude mice were injected with  $10^5$  ( $\bullet$ ),  $10^6$  ( $\circ$ ),  $10^7$  ( $\blacksquare$ ), or  $10^8$  ( $\square$ ) lymph node and spleen cells from chronically infected BALB/c mice and were challenged with  $10^6$  *L. major* promastigotes on day 0. Control groups were not given any lymphocytes ( $\blacklozenge$ ).

ties of resistance-promoting cells in chronically infected mice.

#### Are disease-promoting cells present in infected genetically resistant mice?

We have previously demonstrated that the disease-promoting activity of cells from genetically susceptible BALB/c mice can be increased by injection of a carbohydrate derivative of a host-protective *L. major* glycolipid antigen (Mitchell and Handman, 1986). Among genetically resistant mice, C57BL/6 can become more susceptible to chronic disease provided large amounts of this carbohydrate are injected in Freund's adjuvant. We have therefore examined whether lymphocytes taken from currently infected C57BL/6 mice are less able to promote resistance to infection in syngeneic nude mouse recipients. For this experiment, 2 C57BL/6 mice with lesion scores of 3 and with no sign of healing were chosen as spleen cell donors. These lesions were much larger than those usually seen in euthymic C57BL/6 mice challenged with *L. major* because mice of this strain generally develop only small lesions of score 1 to 2 that heal within 1–2 mo. For comparison, C57BL/6 mice that represented the standard course of infection and had recovered from infection were used and either  $3 \times 10^6$  or

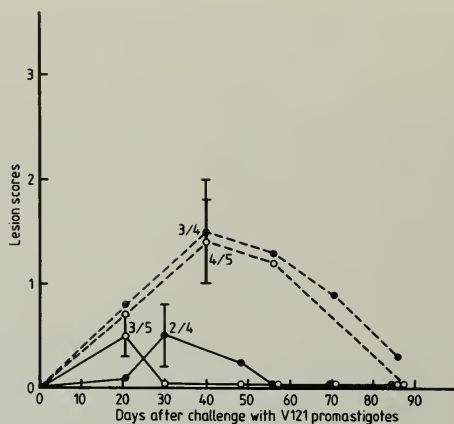


FIGURE 4. Reconstitution of nude mice with T cells from infected genetically resistant mice. On day 0, C57BL/6 nude mice (4 or 5 per group) were injected intradermally with  $10^6$  promastigotes of *L. major* and intraperitoneally with either  $3 \times 10^6$  ( $\circ$ ) or  $3 \times 10^7$  ( $\bullet$ ) spleen cells from euthymic C57BL/6 mice that had recovered from infection (solid lines) or were currently infected with *L. major* (broken lines).

$3 \times 10^7$  spleen cells from each donor type were injected to C57BL/6 nude mice that were challenged with  $10^6$  *L. major* promastigotes. Unless reconstituted with lymphocytes from syngeneic euthymic mice, C57BL/6 nude mice, like nude mice from other strains, are highly susceptible to disease and develop progressing lesions with fatal outcome. The course of lesion development in Figure 4 shows that lymphocytes from currently infected euthymic mice are less able to mediate resistance to infection in nude mice, because lesions are larger and take longer to heal than in nude mice that received cells from recovered euthymic mice.

#### DISCUSSION

The studies reported in this paper supplement 2 other approaches that are also designed to examine the basis of T cell-dependent disease resistance and disease promotion in murine cutaneous leishmaniasis. These other approaches are the subdivision of the L3T4<sup>+</sup> T cell population according to additional surface markers including Ly-24 (Pgp-1) and the functional characterization of the resulting subsets (Moll and Scollay, submitted for publication) as well as the *in vivo* and *in vitro* analysis of T cell responses to defined glycoconjugate antigens of *L. major* (Moll et al., submitted for publication).

The results of the experiments reported here substantiate a conclusion made from other ex-

periments, namely that disease-promoting cells can be induced in genetically resistant C57BL/6 mice (Fig. 4). In addition, we extended the notion that lymphoid organs of genetically susceptible BALB/c mice contain a mixture of disease-promoting and resistance-promoting cells by demonstrating for the first time that even BALB/c mice chronically infected with *L. major* possess resistance-promoting cells. In these mice, disease-promoting cells can be titrated out to uncover the activities of resistance-promoting cells that are still present at a frequency apparently as high as in lymphoid organs of uninfected mice (Fig. 3). The alternative explanation is that the 2 cell types are one and the same, the outcome of infection depending in some way on the numbers of cells present. Against this interpretation is the earlier observation (Mitchell, 1983) that using lymphocytes from severely affected donor mice with late-stage visceralized disease, no cell dose was found to mediate resistance (see also Liew, 1986). Presumably, the balance between numbers of disease-promoting cells and resistance-promoting cells will depend on the duration (severity) of infection and the location from which cells are harvested in chronically infected mice. The proportion of activated T cells with disease-promoting activity appears to be highest in lymph nodes that are draining the site of the lesion (Moll and Mitchell, unpubl. obs.). From the recent study of Locksley et al. (1987), analyses of T helper cells of type 1 and type 2 become critical to the understanding of disease outcome in all such experiments. However, no surface markers to distinguish these L3T4<sup>+</sup> T cell subsets are available as yet.

A new observation is that T cells from donor mice chronically infected with *L. major* do not enhance the rate of lesion development in nude recipients. Thus, in mice given low numbers of parasites, the presence of disease-promoting T lymphocytes does not change the time point of first appearance of skin lesions (Fig. 1). Because during development of the primary infection in genetically susceptible mice, the increase in lesion size reflects the concomitant increase in the number of parasites in the lesions (Hill et al., 1983), it can be concluded that T cell-dependent exacerbation of cutaneous disease is *most unlikely* to be due to accelerated proliferation of amastigotes in the lesion. Rather, the data are consistent with the hypothesis that T cells promote disease by inhibiting the activity of host-protective T lymphocytes. Another new finding

reported in this paper is that the host-protective activity of T cells from uninfected donor mice is extraordinarily potent even in long-term chronically infected nude recipients (Fig. 2). In contrast to the results obtained after inoculation of T cells to chronically infected nude mice, attempts to reverse disease in chronically infected euthymic mice using lymphocyte populations have been unsuccessful (Mitchell, 1983).

Some discussion on the cellular events in C57BL/6 mice infected with *L. major* is warranted. Using a crude *in vivo* limiting dilution assay in nude mice, we have not been able to show a marked increase in the apparent frequency of resistance-promoting cells in recovered hyperimmune mice relative to naive C57BL/6 mice (Mitchell et al., 1987). On the other hand, using the same type of *in vivo* assay, the apparent frequency of disease-promoting cells is increased in chronically infected BALB/c mice and in BALB/c mice immunized with a carbohydrate antigen of *L. major* isolated by affinity chromatography with a monoclonal antibody (Mitchell and Handman, 1986; Mitchell et al., 1987). This same carbohydrate antigen, provided it is injected in high doses and in Freund's adjuvant (and then not in every experiment), can prolong subsequent disease in C57BL/6 mice. We speculate that most genetically resistant mice are nonresponders to this and other disease-promoting antigens but that they can be stimulated to respond under some conditions. In the experiment reported here (Fig. 4), cells from diseased C57BL/6 mice were less able to promote resistance in nude mice than cells from recovered C57BL/6 mice. It becomes important to determine whether, in a vaccination program, some humans are high responders to disease-promoting antigens (like BALB/c mice) and others, though relatively resistant, can respond to such antigens under some circumstances (like C57BL/6 mice).

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## DEVELOPMENTAL VARIATION OF *HETERAXINOIDES XANTHOPHILIS* (MONOGENEA) ON HOSTS OF DIFFERENT SIZES

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**ABSTRACT:** *Heteraxinoides xanthophilis*, a gill parasite of the spot, *Leiostomus xanthurus*, has an asymmetrical triangular-shaped haptor with multiple clamps situated along its edges. Clamps are usually attached consecutively to successive gill lamellae of the host. Worms accommodate during development to the gills of the particular size host. The number of clamps/mm along the haptor decreased with increased host size. Worms collected from 3 size classes of fish were significantly different in overall size at all stages of development. Clamps develop sequentially, with the younger, and eventually larger, clamps being anterior. Each successive clamp reaches an ultimate size determined by host size. The various stages (as indicated by the number of clamps present) at which the larval haptor is lost or the reproductive system develops are not affected by host size.

The effect of host size on the development of monogeneans has received little study. Paling (1965) found small specimens of *Discocotyle sagittata* (Leuckart, 1842) on juvenile *Salmo trutta* Linnaeus and larger worms on adults, but indicated that this could be attributed to continued growth of worms during a span of several years. Gussev and Kulemina (1971a, 1971b) found that body size, sclerotized armature, and copulatory complex varied with host size in several monogeneans of the genera *Dactylogyrus* Diesing, 1850, and *Tetraonchus* Diesing, 1858, and in multiple clamped diclidophorid *Diplozoon megan* Bychowsky and Nagibina, 1959. They attributed much of this variation to gill lamellar thickness associated with host size. Lengths of the median anchors of *D. megan*, which develop in the oncomiracidium before hatching and infestation, are not dependent on host size as would be expected (Gussev and Kulemina, 1971a). Cone and Burt (1985) showed that the ancycrocephaline *Urocleidus adspectus* Mueller, 1936, grew larger on larger hosts and conclusively ruled out the idea that larger worms were older worms. Thoney (1986) found that the body length and posterior clamp width of *Microcotyle sebastis* Goto, 1890, were smaller in worms collected from small experimentally infested juvenile black rockfish than in worms from naturally infested adult fish. This phenomenon may be widespread throughout the Monogenea, but many more species must be examined before its significance and extent are documented. The relationship between worm development and host size in feral populations is documented below for the first time for a species

of the subfamily Microcotylinae—*Heteraxinoides xanthophilis* and its host *Leiostomus xanthurus*.

### MATERIALS AND METHODS

One hundred and two spot, ranging from 33 to 176 mm standard length (SL), were collected from the Pamlico River, North Carolina (35°20'N, 76°30'W), the York River, Virginia (37°15'N, 76°30'W), and continental shelf waters of the Atlantic coast U.S.A. from the Delaware Bay (39°N) to Cape Fear, North Carolina (34°N). Fish were either frozen or fixed in 10% neutral buffered formalin until necropsied. Worms were stained with Van Cleave's hematoxylin, mounted in Permount, and measured using a calibrated ocular reticle. The width of clamps was measured and used as an index of clamp size, because the width did not vary with the state of contraction of either the clamp or the worm as a whole.

Some infested gills were embedded in paraffin wax, sectioned, and stained with Harris' hematoxylin and eosin. The number of gill lamellae/mm was determined by making several counts of lamellae along filaments from various locations along the gill arch. A mean number of lamellae/mm on a filament was then calculated for each fish. Because growth, as determined by distance, between adjacent lamellae was consistent among arches (Roubal, 1987), only lamellae on the first arch were examined in this study.

Model I linear regressions (Sokal and Rohlf, 1969) were used to test whether the number of clamps on the longer side of the haptor (Fig. 1) could be used to estimate the total lengths of monogeneans from 3 groups of fish—(A) 131–176, (B) 61–130, and (C) 33–60 mm SL. Procedures outlined by Snedecor and Cochran (1980) were then used to test for differences in the slopes and heights of the linear regressions. Linear regression and correlation analyses (Sokal and Rohlf, 1969) were used to analyze interrelationships among other characters measured or counted.

### RESULTS

The length of *Heteraxinoides xanthophilis* from any size class of fish was related ( $P < 0.05$ ) to

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FIGURE 1. Posterior of *Heteraxinoides xanthophilis* showing clamps on the long side (L) and short side (S) of the haptor. Scale bar = 0.1 mm.

the corresponding number of clamps along the longer side of the haptor (Fig. 2). Worms collected from the gills of smaller fish were significantly smaller ( $P < 0.05$ ) than those collected from larger fish (Fig. 2).

Gill lamellae were farther apart as host size increased ( $P < 0.05$ ) (Fig. 3). Clamp spacing on the haptor of *H. xanthophilis* also increased with host size ( $P < 0.05$ ) (Fig. 3). The clamps on the haptor usually attached consecutively to the distal tips of each successive lamella (Fig. 4).

Posterior clamps developed first. New clamps developed anterior to older clamps in a sequential fashion. The width of the posterior clamps of worms collected from a particular size class of fish did not increase as worms grew, and posterior clamp width was independent of the number of clamps per worm ( $P > 0.05$ ) (Fig. 5). Posterior clamp width was greater in worms from larger hosts ( $P < 0.05$ ) (Fig. 6).

The largest clamps were usually located half way along the haptor. Anterior clamps that had completed development were as large or larger than those more posterior. Therefore, the width

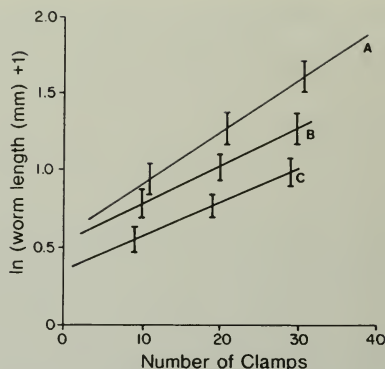


FIGURE 2. Length of worm as a function of the number of clamps on the long side of the haptor in *Heteraxinoides xanthophilis* collected from fishes 131–176 mm SL (line A;  $y = 0.542 + 0.036x$ ;  $r^2 = 0.73$ ;  $n = 23$ ), from fishes 61–130 mm SL (line B;  $y = 0.546 + 0.024x$ ;  $r^2 = 0.34$ ;  $n = 48$ ), and from fishes 33–60 mm SL (line C;  $y = 0.354 + 0.022x$ ;  $r^2 = 0.46$ ;  $n = 31$ ). Bars represent 95% confidence limits.  $\ln$  [length (worm) (mm) + 1] = natural logarithm of [length (worm) (mm) + 1].

of the largest clamps increased with the number of clamps per worm ( $P < 0.05$ ;  $r = 0.62$ ). However, the greatest clamp width also increased with host size ( $P < 0.05$ ;  $r = 0.52$ ).

The larval haptor was lost following the development of 5 or 6 pairs of clamps. Following acquisition of 6 or 7 pairs of clamps, the haptor of *H. xanthophilis* becomes asymmetrical as one side develops clamps more rapidly than the other. For example, the longer and shorter side can have up to 39 and 29 clamps, respectively. The testes also began to develop after development of 7 pairs of clamps. The ovary became apparent

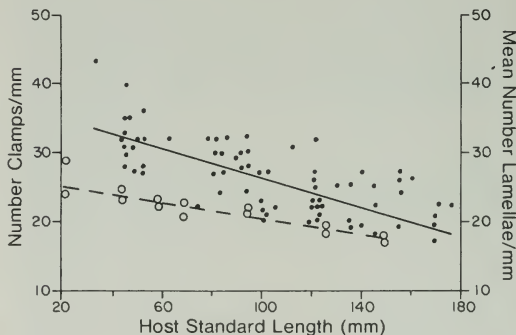


FIGURE 3. The number of clamps/mm on the haptor of *Heteraxinoides xanthophilis* (solid line and solid circles;  $y = 37.2 - 0.11x$ ;  $r^2 = 0.64$ ;  $n = 103$ ) and the mean number of lamellae/mm on gill filaments of *Leiostomus xanthurus* (dashed line and open circles;  $y = 26.5 - 0.054x$ ;  $r^2 = 0.73$ ;  $n = 14$ ) as a function of host standard length.



FIGURE 4. Haptor of *Heteraxinoides xanthophilis* attached to the gill lamellae of *Leiosomus xanthurus* (some clamps are not shown in this section). Scale bar = 0.25 mm.

when 13–15 clamps had developed along the long side of the haptor (LSH) and, by the time 17 clamps had developed, some worms produced eggs. The stages at which these events occurred did not depend on host size. Egg length was variable ( $\bar{x} = 0.171$ ;  $0.093$ – $0.207$  mm) and was not affected by host size.

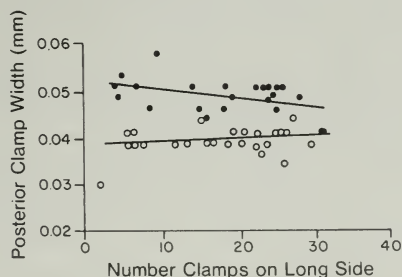


FIGURE 5. Posterior clamp width as a function of the number of clamps on the long side of the haptor of *Heteraxinoides xanthophilis* collected from fishes 33–60 mm SL [open circles;  $y = 0.038 + (8.1 \times 10^{-5})x$ ;  $r^2 = 0.06$ ;  $n = 25$ ] and from fishes 100–130 mm SL [closed circles;  $y = 0.051 + (-1.4 \times 10^{-4})x$ ;  $r^2 = 0.11$ ;  $n = 22$ ].

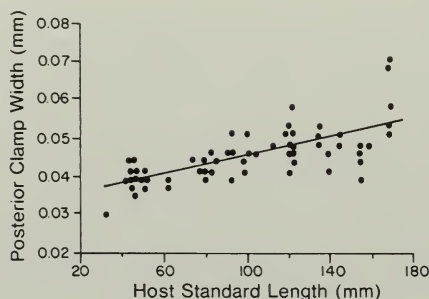


FIGURE 6. Posterior clamp width of worm as a function of host standard length ( $y = 0.039 + 0.005x$ ;  $r^2 = 0.67$ ;  $n = 87$ ).

## DISCUSSION

The number of haptor clamps present in *Heteraxinoides xanthophilis* is a better indicator of a worm's stage of development than body length because variable states of contraction at fixation increase variability of body length, and host size affects the size of worms at all stages of development. Thoney (1986) concluded the same for *Microcotyle sebastis*.

Relative increments of growth were similar for worms from all size hosts as indicated by the similar slopes of the regression lines (Fig. 2). However, the heights of the lines are different, indicating that some factor, e.g., gill size, probably affects the size of worms at all stages of their development. Larger fish had larger worms. For example, a worm with 30 clamps (LSH) from the largest size class of fish had a mean length of 3.7 mm, whereas a worm with 30 clamps from the smallest size class of fish was only 1.7 mm long. These data also suggest that the youngest worms collected from each size class of hosts over the season had already adjusted to that size of host. In fact, worms with 2 pairs of clamps from hosts of all size classes had larger clamps in larger hosts. The stimuli affecting development must act soon after the developing worm attaches to the host. The fact that external stimuli can affect the development of worms has been demonstrated by Ogawa and Egusa (1981) in the closely related heteraxinid, *Heteraxine heterocerca*.

Lamellae were spaced farther apart in larger individuals of the host *L. xanthurus* (Fig. 2), as well as of other fish species (Hughes, 1984). As new clamps develop anteriorly they must be spaced to allow attachment to each successive lamella. It is theoretically possible that spacing of clamps along the haptor may accommodate to spacing of lamellae in a particular sized host



by stretching or contraction of tissue between the clamps. However, it is more likely that the haptor must grow to accommodate to gills as the host grows. This is supported by the fact that the ratio of haptor length to body length does not change with size of the host, indicating that the entire worm is larger on larger hosts.

Lamellae were also longer and wider in larger *L. xanthurus*. Lamellae have also been found to increase in size with host size in several other fishes (Muir and Hughes, 1969; Hughes, 1984; Hughes et al., 1986). Because the size of clamps must accommodate to lamellae to permit attachment, their size must also be dependent on host size.

Figure 3 shows that on any size host there were fewer lamellae/mm on a filament than clamps/mm for worms from that size host. Worms attach to the distal tips of the lamellae and tend to pull the tips toward the center of the haptor. For example, there are 25 clamps/mm on the haptor of the specimen depicted in Figure 4 and only 20 lamellae/mm on the gill filament (measured at the bases of the lamellae), yet each clamp is generally attached to a separate lamella.

The clamp's size must be large enough to grasp lamellae but not be too large for a young worm to manipulate and support. These 2 factors, and not worm age, determine the ultimate size of posterior clamps, because the width of the posterior clamps of worms was independent of the number of clamps per worm (Fig. 5). As juveniles add clamps and increase in overall size they are able to accommodate larger clamps, and because worms are larger on larger hosts, both worm age and host size affect the ultimate size of the more anterior clamps. Each successive clamp appears to reach an ultimate size with the younger and eventually larger ones being more anterior.

The stages at which the larval haptor is lost and various reproductive structures develop are not dependent on host size. For example, testicular development began following development of 6–7 pairs of clamps in worms from both a 33- and 150-mm SL fish. This phenomenon suggests that the developmental sequence of events during growth is not variable, but the sizes attained by several anatomical features are variable. Egg size is highly variable even within an individual worm and therefore is independent of host size. Possible effects that egg size may have on size of the oncomiracidium at hatching are unknown.

It is not known how long these worms live, but the hosts increase in size rapidly from about

35 mm SL in May until they leave the estuary at about 100 mm SL in October or November. It is likely that various features of the worms, such as the spacing of clamps, overall size of worms, or the sizes of clamps (that have not yet reached their ultimate size), continue adjusting to gills of these rapidly growing fish.

Variation in the size of taxonomically important features can create complex taxonomic problems when studying individuals of a single species or of closely related species on different hosts, especially if the hosts vary in size and/or gill structure. Therefore, it is important that parasites from all host species and/or a wide range of sizes of a particular host be examined carefully when defining taxa or studying morphological relationships among species.

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## HEAT SHOCK-LIKE POLYPEPTIDES OF THE SPOROZOITES AND MEROZOITES OF *EIMERIA BOVIS*

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**ABSTRACT:** Heat shock proteins (hsps) are a group of highly conserved polypeptides found in a wide variety of organisms. Polypeptides of sporozoites and merozoites of *Eimeria bovis*, blotted onto nitrocellulose, were probed with antibodies to artificially constructed peptides representing portions of the cDNA-generated fragment of pf75, the 75K hsp of merozoites of *Plasmodium falciparum*. Polypeptide antigens of sporozoites and merozoites of *E. bovis* with molecular weights of 46K, 71-72K, and 75K reacted with antibodies against pf75, indicating that they are hsp70 (the 70K family of hsps) or hsp70 cognates (noninducible proteins homologous to hsps). Radiolabeling with <sup>125</sup>I and treatment with antibodies against pf75 detected a 71K antigen on the merozoite surface. Hsps in sporozoites of *E. bovis* are either constitutive or evoked by treatment at 37 C for *in vitro* excystation. If hsp70 is mandatory for parasite survival, it may prove to be an appropriate antigen for a vaccine against bovine coccidiosis.

All organisms are subject to environmental temperature fluctuations. Correlated with heat stress is the appearance, primarily in the nucleus, of a small group of highly conserved polypeptides, the heat shock proteins (hsps). Hsps have been found in all genera in which they have been sought (Lindquist, 1986). Hsps have been detected in several parasitic protozoa including *Trypanosoma brucei*, *Leishmania major* (Van der Ploeg et al., 1985), *T. cruzi* (Dragon et al., 1987), and *Plasmodium falciparum* (Ardeshir et al., 1987). In these 2-host parasites, hsps may facilitate the parasite's transference from existence at the lower temperature in the insect host to the higher temperature of the mammalian host.

Despite similarities in amino acid sequences in the hsps of hosts and parasites, different conditions may be required for hsp evocation. Differential hsp expression may selectively induce host immune responses against parasite hsp antigens. Also, sufficient lack of homology between parasite and host hsps may permit the host immune response to selectively attack the parasite hsp's, especially if hsps are on the parasite surface. In this paper, we report the presence of hsp or hsp cognates in both the sporozoites and merozoites of *Eimeria bovis*, a pathogenic coccidian of cattle. Hsp cognates are constitutive noninducible proteins that are homologous to hsps (Craig, 1985).

### MATERIALS AND METHODS

#### Preparation of sporozoites and merozoites

Oocysts of *E. bovis* were collected from the feces of experimentally infected Holstein-Friesian calves, and the sporozoites were excysted and purified as previously described (Fayer and Hammond, 1967; Larsen et al., 1984). First-generation merozoites of *E. bovis* were obtained from cultured bovine monocytes that had been inoculated 14-18 days earlier with sporozoites (Reduker and Speer, 1986, 1987).

#### SDS-PAGE, western blots, ELISA

Purified whole sporozoites or merozoites were solubilized in SDS-solubilizing solution (Reduker and Speer, 1986) and samples containing 1.2, 6, and 15 × 10<sup>6</sup> organisms were resolved on 0.75-mm-thick 12.5% polyacrylamide gels (Wong et al., 1979). Parasite proteins in gels were electroblotted onto nitrocellulose sheets (Towbin et al., 1979) using a BioRad trans-blot apparatus (BioRad, Richmond, California) at 30 V for 20 hr. Residual polypeptides on gels were visualized with silver stain (Switzer et al., 1979; Merrill et al., 1981). The entire spectrum of polypeptides blotted onto the nitrocellulose was visualized by reversible staining with Ponceau red (James Etchison, pers. comm.).

Rabbit antisera generated against specific sequences of synthetic peptides of the *Plasmodium falciparum* hsp75 (i.e., a 75K hsp) molecule (Richman and Reese, 1988; Richman et al., 1988) were applied to polypeptides of merozoites or sporozoites of *E. bovis* blotted onto nitrocellulose. The antisera used were: (1) anti-repeat, which is against amino acid residues 212-227 near the carboxy terminus of pf75, at a 1:750 dilution in BLOTTO (Johnson and Elder, 1983); (2) anti-28 that reacts against a 28-mer in a major helical region that includes residues 104-129; and (3) anti-64 and -76 that were prepared against peptides between anti-repeat and anti-28 and extending from amino acids 141 to 204 and from 129 to 204, respectively. The anti-28, -64, and -76 sera were all used at a 1:3,000 dilution in BLOTTO.

Parasite polypeptides on nitrocellulose were blocked with BLOTTO, exposed to 1 of the above antisera for

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18 hr at 4 C, rinsed with BLOTTO, and treated with the second antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG; Boehringer-Mannheim, Indianapolis, Indiana). The reaction color was developed with 4-chloro-1-naphthol (Hawkes et al., 1982). Preimmune rabbit serum was used as above as a control.

To assure that all hsp70-like molecules were detected by ELISA, sporozoite or merozoite polypeptides on nitrocellulose were treated with 2 higher concentrations of each antiserum. Anti-repeat serum was used at dilutions of 1:100, 1:250, and 1:750; the other 3 antisera were each used at dilutions of 1:250, 1:1,000, and 1:3,000 in BLOTTO.

#### Surface labeling with [ $^{125}$ I]lactoperoxidase

About  $7.5 \times 10^6$  merozoites were surface labeled with 250  $\mu$ Ci  $^{125}$ I (New England Nuclear, Boston, Massachusetts) (Morrison, 1980), solubilized in SDS solubilizing solution (Reduker and Speer, 1986), and electrophoretically separated on a 12.5% polyacrylamide gel. The resolved polypeptides were blotted onto nitrocellulose and probed with anti-repeat serum, and autoradiographs of the blots were made with Kodak X-Omat AR film with 1.5 hr exposure at room temperature.

#### Indirect immunofluorescence assay (IFA)

Sporozoites and merozoites were air-dried to 8-well microscope slides, with or without a 10-min acetone fixation, treated with various concentrations of antisera (i.e., from undiluted to 1:2,000 in Dulbecco's phosphate-buffered saline, PBS), rinsed in PBS, and treated with fluorescein-conjugated goat anti-rabbit IgG (Boehringer-Mannheim, Indianapolis, Indiana) at a 1:40 dilution in PBS. To minimize nonspecific fluorescence, aliquots of the fluorescein-conjugated antibody were preabsorbed with sporozoites or merozoites that had been solubilized in SDS and conjugated to cyanogen bromide-activated sepharose (Hudson and Hay, 1980). Controls consisted of sporozoites or merozoites treated with preimmune serum and then fluorescein-conjugated IgG antibody or fluorescein-conjugated IgG antibody only.

## RESULTS

#### Western blot-ELISA

Antibodies in the various antisera generated against pf75 peptide fragments reacted with 3 hsp70-like antigens in sporozoites and merozoites of *E. bovis* that had approximate relative molecular weights ( $M_r$ ) of 46K, 71–72K, and 75K (Fig. 1). None of the hsp-like antigens appeared to be specific for either merozoites or sporozoites. In both sporozoites and merozoites, the most intense reactions occurred with the anti-repeat serum. The 71–72K band was the most prominent, followed by the 46K band. The second most reactive antiserum was anti-28-mer, which reacted with both the 71–72K and the 75K bands in both stages. Anti-76-mer and anti-64-mer antisera gave faint reaction products with

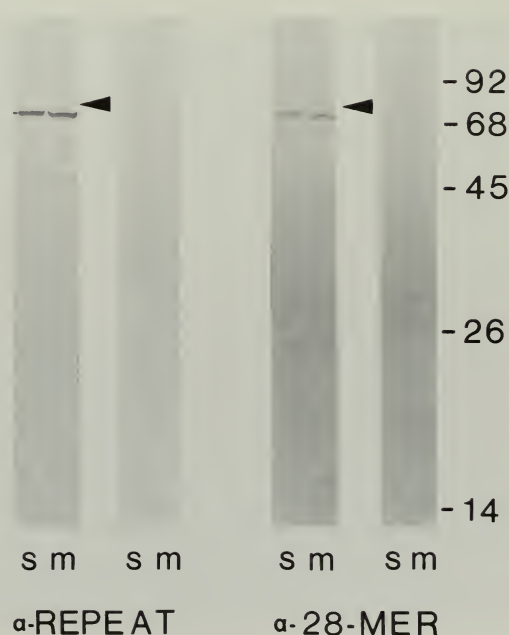


FIGURE 1. Western blot analysis of sporozoites (s) and merozoites (m) of *E. bovis* probed with anti-repeat and anti-28-mer rabbit sera. Single arrows indicate the 75K polypeptide. The control antisera are at the right of each set. Approximate molecular weight standards ( $\times 10^3$ ) are shown at the right.

the sporozoite 71–72K antigen (data not shown). Increasing antiserum concentrations did not alter the patterns of hsp70-like antigens just described for the higher dilutions.

In summary, the order of reactivity of the 4 antisera, from strongest to weakest, was anti-repeat, anti-28-mer, anti-76-mer, and anti-64-mer.

#### Surface labeling with $^{125}$ I

Blots of  $^{125}$ I-labeled merozoites, probed with anti-repeat antiserum, displayed a 71–72K antigen with the peroxidase conjugate (data not shown). Autoradiographs of the blots that were exposed for 1.5 hr exhibited a prominent 71–72K antigen (Fig. 2).

#### Immunofluorescence

When treated with a 1:250 dilution of anti-repeat serum, air-dried merozoites exhibited intense fluorescence (Figs. 3, 4), whereas those fixed in acetone did not fluoresce.

Sporozoites that were air-dried with or without acetone treatment displayed autofluorescence when they were exposed to preimmune serum only or to anti-repeat serum followed by fluorescein-conjugated goat anti-rabbit IgG anti-



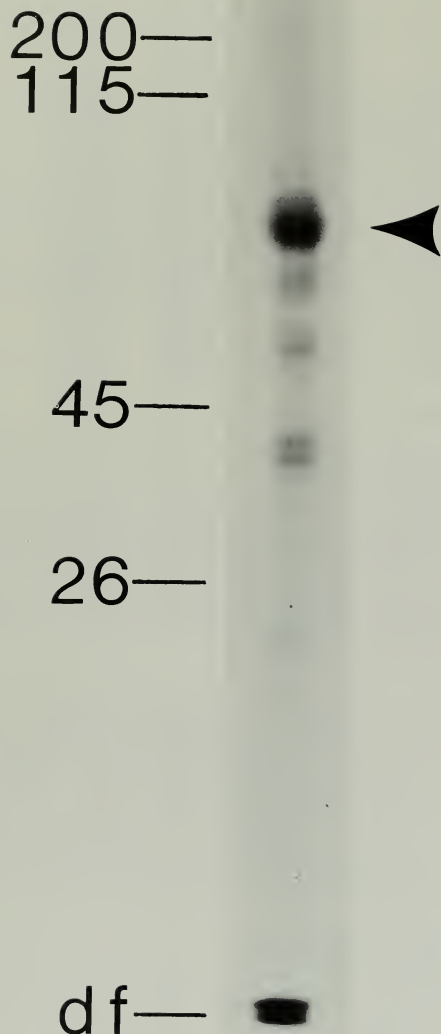


FIGURE 2. Autoradiogram of a western blot of  $^{125}\text{I}$  surface-labeled first-generation merozoites of *E. bovis*. Arrow points to 71–72K surface-labeled antigen. The blot was probed with anti-repeat serum that reacted with the 71–72K band only (not shown). Molecular weight standards ( $\times 10^3$ ) and dye front (df) are shown at the left.

body. Similar results were obtained with fluorescein-conjugated IgG antibody that had been preabsorbed with solubilized sporozoites.

#### DISCUSSION

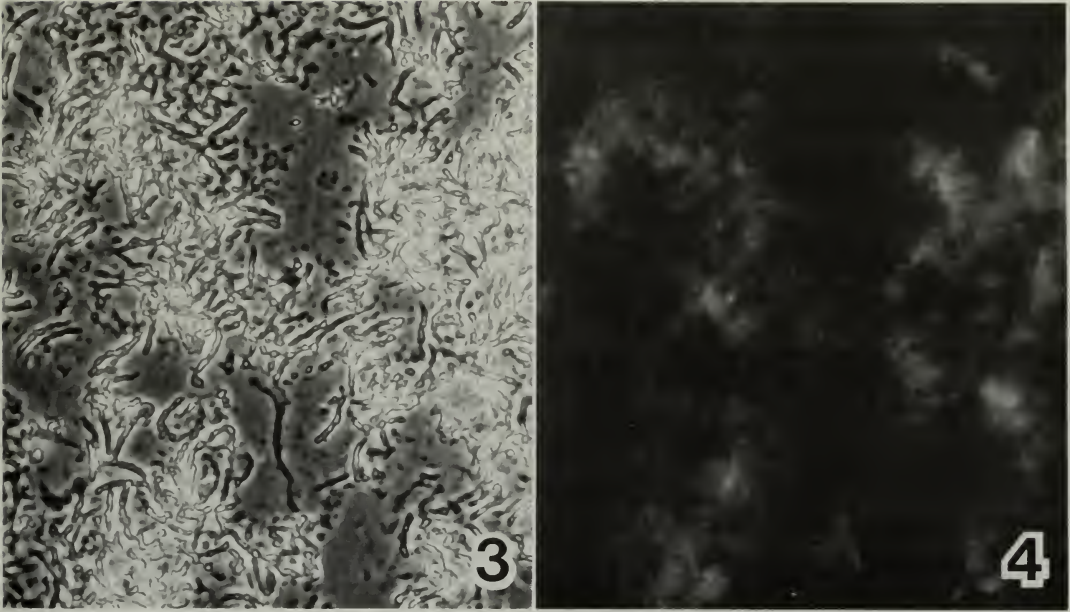
Most of the reports concerning hsps have involved organisms that belong to genera (e.g.,

*Drosophila*, *Saccharomyces*, *Escherichia*) other than those of the parasitic protozoa. Consequently, there are a few reports on hsps in protozoan parasites and most of these have appeared during the last 5 yr. In the present study, by using anti-serum directed against hsps in *P. falciparum* we found that both sporozoites and merozoites of *E. bovis* contain at least 3 hsp-like antigens. Two of the hsp-like antigens of *E. bovis* had relative molecular weights of 71–72K and 75K, which occur within the molecular weight range (i.e., 68–76K) of the most common hsps of other genera. The third hsp-like antigen of *E. bovis* had an  $M_r$  of 46K, which is slightly larger than actin-sized (i.e., 43K) hsps reported for some genera (Silver et al., 1983). Hsps of 20K or 83–90K, which are common to other organisms, were not detected in *E. bovis*, indicating that such hsp-like antigens were absent or do not contain epitopes common to the 43K, 71–72K, and 75K hsp-like antigens. Antisera against the 20K and 83–90K hsps will be needed to determine if such hsps occur in *E. bovis*.

[ $^{125}\text{I}$ ]lactoperoxidase labeling of live merozoites of *E. bovis* indicated that the 71–72K antigen was present on the parasite surface. Air-dried, but not acetone-fixed, merozoites of *E. bovis* displayed immunofluorescence using anti-repeat serum. Acetone may have caused sufficient alteration in the epitopes in the 71–72K antigen in the merozoite plasmalemma to interfere with their ability to react with antibodies in the anti-repeat serum.

By using  $^{125}\text{I}$ -labeling, Whitmire et al. (1988) found 6 prominent proteins on the surface of sporozoites of *E. bovis* of which the largest protein was 54,900K. Thus, sporozoites may lack the 71–71K surface antigen found in merozoites of *E. bovis*. Hsp75 occurs in erythrocytic forms and on the surface of erythrocytic merozoites of *P. falciparum* (Ardeshir et al., 1987), but whether hsps are present in the sporozoite is unclear. Hsp70-like antigens on the surface of merozoites of *E. bovis* might warrant investigation as candidate vaccine antigens provided similar antigens are not expressed simultaneously by the host.

In *Trypanosoma brucei* and *Leishmania major*, 2 hsps (70K, hsp70; 83K, hsp83) were reported to be more abundant in the mammalian host form than in the insect vector form (Van der Ploeg et al., 1985), but no other hsp size classes were reported. Elevation in hsp70 and hsp83 production in the amastigote over that of the promastigote may be an adaptation to the warm-blooded mammalian host. In contrast,



FIGURES 3, 4. Photomicrographs. 3. Phase-contrast photomicrograph of air-dried merozoites of *E. bovis*.  $\times 800$ . 4. Photomicrograph of immunofluorescent assay reaction of anti-repeat antiserum with same specimens as in Figure 3.  $\times 800$ .

stages of *Trypanosoma cruzi* that occur in the insect host and those that occur in the mammalian host exhibit an hsp85-like molecule, indicating that it is constitutively produced (Dragon et al., 1987). It is not known whether hsp85 is a true hsp or an hsp cognate (Craig, 1985). The cellular locations of hsps in *T. brucei*, *L. major*, and *T. cruzi* are unknown.

If the hsp-like antigens found in *E. bovis* are actually hsps, they may need to be present in both stages as a safeguard for the sporozoite against temperature fluctuations outside the host and against the high body temperature of the bovine host experienced by both sporozoites and merozoites. It should be noted that excystation of sporozoites from sporocysts of *E. bovis* requires a 2.5–3.5-hr incubation at 37°C in excysting fluid. If it is not feasible to excyst sporozoites at lower temperatures, then unexcysted sporozoites maintained at lower temperatures should be examined for hsp70 cognates. The hsp-like antigens of *E. bovis* may be hsp70 cognates that are constitutively produced molecules and may have amino acid sequences that are homologous with hsp70, which is known to be biologically important in other organisms (Craig, 1985). In contrast to hsps, production of hsp

cognates is not augmented by temperature shifts (Craig, 1985; Lindquist, 1986).

High temperature shifts will induce fibroblasts to produce hsp72, which is found mainly in the nucleolus (Welch and Suhan, 1985). In contrast, murine NIH/3T3 hsp72 appears to be constitutive at low and high temperatures (Hughes and August, 1982). Hsp74 (i.e., 74K) in *Drosophila*, which is slightly smaller than the *E. bovis* hsp-like 75 antigen, has been shown to be associated with the karyoskeleton at high temperatures where it may be essential for maintaining karyoskeletal stability (McConnell et al., 1987). In the fungus *Achyla*, hsp74 is primarily a cytoplasmic polypeptide (Silver et al., 1983). A direct comparison of the sequences of hsp74 from these divergent species is not available. Likewise, we have not determined whether the *E. bovis* 71–72K antigen is constitutive or inducible, nor do we know of its cellular location except that it occurs on the merozoite surface.

The 46K antigen of *E. bovis* is most akin to hsp43 in size. In *Achyla*, hsp43 is mainly a nuclear protein and migrates from the cytoplasm to the nucleus during heat shock (Silver et al., 1983). Despite its similarity in size to actin, hsp43 is distinguishable by being more acidic (Silver et



al., 1983). The properties and cellular locations of the 46K antigen, as well as other hsp homologs of *E. bovis*, await further analysis.

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## BLOOD PARASITES OF BIRDS IN CAMEROON

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**ABSTRACT:** Birds from south-central Cameroon, western Africa, were surveyed for blood parasites from August to October 1986. Of 331 birds examined, representing 65 species of 15 families and 6 orders (mostly passerines), 55 (17%) were found to be infected with 1 or more genera of hemotropic parasites. These included: *Haemoproteus* spp. (11% prevalence), *Leucocytozoon* spp. (3%), *Plasmodium* spp. (2%), *Trypanosoma* spp. (1%), and microfilariae of filariid nematodes (1%). Several new host-parasite associations were identified.

Relatively little recent attention has been given to the study of hemotropic parasites of birds in West Africa (see Bray, 1964; Herman et al., 1976; and Bennett et al., 1981, for bibliographies). Most recent studies have been conducted in East Africa. We are not aware that any surveys of avian hematozoa have been conducted in Cameroon, and we are aware of just 3 published surveys of birds in adjoining countries: 2 in Nigeria (Ashford, 1971; Smith and Cox, 1972) and 1 in Chad (Williams et al., 1977). The 2 Nigerian surveys were limited to Palearctic migrants.

As part of an ecological study on an estrildid finch (*Pyrenestes ostrinus*) in Cameroon (Smith, 1987), we conducted a preliminary survey of the hematozoa of various birds captured over a 3-mo period. The purpose of the study was to record the prevalence of blood parasites in some rarely examined host species and to identify promising directions for research into the epizootiology of hematozoan infections in Cameroon birds. We report here on the results of that survey and record some newly recognized host-parasite associations.

### MATERIALS AND METHODS

Birds were captured in mist nets in seasonally flooded swamp forest along the Nyong River in south-central Cameroon, 130 km southeast of Yaoundé (3°14'N; 12°11'E, 600 m elevation). The study took place from August to October 1986, immediately prior to and during the major rainy season. Additional characteristics of the study site are given elsewhere (Letouzey, 1968; Smith, 1988). Blood was sampled from the majority of birds by clipping a claw. As part of a concurrent study, 18 (6%) of the sampled birds were killed; blood was taken from the body cavities of these birds immediately after death. Smears were made, air-dried, and fixed in methanol in the field, and they were stained

in Giemsa solution in a laboratory 1–2 wk later. Smears were coverslipped and scanned under darkfield illumination at 200× total magnification, for at least 10 min. *Plasmodium* spp. and *Haemoproteus* spp. are detected readily by this method, due to their brightly refractive hemozoin granules. In the same way, smears also were read under brightfield illumination, which allowed rapid detection of the larger hemoparasites, viz. *Leucocytozoon* spp. and microfilariae of filariid nematodes. Furthermore, smears were scanned for at least 10 min at 400× magnification under brightfield illumination. If a parasite was detected by any of these methods, it was viewed at 1,000× total magnification under oil-immersion brightfield microscopy for confirmation and specific identification. Parasitemias were estimated according to the method of Godfrey et al. (1987) by actually counting 2,000 erythrocytes. Thus, only parasitemias  $\geq 0.05\%$  were quantified. Avian taxonomy and nomenclature conform to those of Louette (1981) and the American Ornithologists' Union (1983). New host-parasite records are claimed based on their absence in the host-parasite checklist of Bennett et al. (1982) and in subsequently published studies (Peirce, 1984a, 1984b, 1984c, 1984d, 1984f).

Representative smears have been deposited in the International Reference Centre for Avian Haematozoa, Memorial University, St. John's, Newfoundland, Canada A1B 3X9, and they have been assigned accession numbers 99420–99437.

### RESULTS

A total of 331 birds, consisting of 65 species from 15 families and 6 orders, was examined for blood parasites (Table I). All of the avian species sampled were either intra-African migrants or nonmigratory. Infections with 1 or more genera of parasites were detected in 55 (17%) of them. Infections with multiple parasite genera were found in 5 (9%) of the infected birds. Parasitemias were  $< 0.05\%$  in 80% of the infected birds. The highest parasitemia of *Haemoproteus* spp. found was 0.65% for *H. sequeirae* in a *Nectarinia olivacea*, and the highest parasitemia of *Plasmodium* spp. encountered was 0.7% for *P. vaughani* in a *Euplectes macrourus*. Only 20% of the parasitemias could be quantified; the re-

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TABLE I. Prevalences of blood parasites in some birds from Cameroon.

Host order, family, and species	Number infected/ number examined (%)		Number infected with:*				
			H	L	P	T	M
COLUMBIFORMES							
Columbidae							
<i>Turtur tympanistria</i>	1/3	(33)	1				
PASSERIFORMES							
Pycnonotidae							
<i>Pycnonotus gracilis</i>	2/8	(25)		2			
<i>Pycnonotus virens</i>	1/37	(3)	1				
Muscicapidae							
<i>Hylia prasina</i>	1/1	(100)	1				
<i>Prinia bairdii</i>	2/5	(40)	2				
Nectarinidae							
<i>Anthreptes collaris</i>	1/2	(50)			1	1	
<i>Nectarinia chloropygia</i>	1/3	(33)	1			1	
<i>Nectarinia olivacea</i>	6/9	(67)	2	2	2	1	1
Ploceidae							
<i>Euplectes afer</i>	2/14	(14)	1				1
<i>Euplectes ardens</i>	2/6	(33)	2				
<i>Euplectes macrourus</i>	2/5	(40)	1		1		
<i>Ploceus aurantius</i>	4/20	(20)	1		3		
<i>Ploceus nigerrimus</i>	6/9	(67)	4	3			
<i>Ploceus nigricollis</i>	2/3	(67)	2				
<i>Quelea erythrops</i>	4/11	(36)	1	3			1
Estrildidae							
<i>Estrilda astrild</i>	1/12	(8)	1				
<i>Estrilda atricapilla</i>	2/5	(40)	2				
<i>Hypargos nitidulus</i>	2/2	(100)	2				
<i>Lonchura bicolor</i>	3/7	(43)	3				
<i>Lonchura cucullata</i>	2/3	(67)	2				
<i>Pyrenestes ostrinus</i>	5/32	(16)	5				
<i>Spermophaga haematina</i>	3/11	(27)	3				
Other species†	0/124						
Totals‡	55/331		36	10	8	3	3
(%)	(17)		(11)	(3)	(2)	(1)	(1)

\* Key: H = *Haemoproteus*; L = *Leucocytozoon*; P = *Plasmodium*; T = *Trypanosoma*; M = microfilariae of unidentified filariid nematodes.

† Host species in which no parasites were found (n): CAPRIMULGIFORMES, Caprimulgidae: *Macrodipteryx vexillarius* (1); COLUMBIFORMES, Columbidae: *Turtur afer* (2); CORACIIFORMES, Alcedinidae: *Alcedo leucogaster* (1), *Ceyx picta* (12), *Halcyon senegalensis* (2); Meropidae: *Merops variegatus* (6); CUCULIFORMES, Cuculidae: *Chrysococcyx caprius* (2), *C. cupreus* (1), *C. klaas* (1); PASSERIFORMES, Estrildidae: *Amandava subflava* (8), *Estrilda melpoda* (3); Fringillidae: *Serinus mozambicus* (1); Hirundinidae: *Hirundo semirufa* (3), *H. senegalensis* (1); Muscicapidae: *Artomyias fuliginosa* (1), *Cossypha niveicapilla* (1), *Fraseria cinerascens* (1), *Muscicapa striata* (2), *Myioparus plumbeus* (1), *Platysteira castanea* (3), *P. cyanea* (3), *Terpsiphone rufiventer* (1), *T. viridis* (1), *Acrocephalus rufescens* (2), *Apalis sharpii* (1), *Camaroptera brachyura* (12), *C. supercilialis* (3), *Cisticola erythrops* (1), *C. galactotes* (7), *Prinia leucopogon* (6), *Sylvietta virens* (6); Nectarinidae: *Nectarinia batesi* (3), *N. cuprea* (1), *N. reichenbachii* (1); Pycnonotidae: *Chlorocichla simplex* (2), *Nicator chloris* (1), *Andropadus latirostris* (3), *Pycnonotus barbatus* (1); Sturnidae: *Lampornis splendidus* (1); PICIFORMES, Capitonidae: *Pogoniulus bilineatus* (6), *P. scolopaceus* (5), *P. subsulphureus* (2); Picidae: *Campethra caroli* (3).

‡ Prevalence of infections with individual parasite genera exceed overall prevalence of parasitism due to multiple infections in some host individuals.

mainder was below 0.05% (i.e., 1 parasitized cell/2,000 cells examined).

The most commonly recognized parasites were *Haemoproteus* spp., including *H. quelea* in weavers (Ploceidae), *H. orizivora* in waxbills (Estrildidae), *H. otocompsae* in a bulbul (Pycnonotidae), *H. sequeirae* in sunbirds (Nectarinidae), and *H. wenyoni* in warblers (Muscicapidae). *Leucocytozoon* spp. identified included *L. majoris* in a sunbird and in weavers and *L. fringillinarum* in weavers. Subgenera of *Plasmodium* spp. identified were *Novyella* and *Giovannolaia* in sunbirds. *Plasmodium vaughani* and *P. circumflexum* were recognized in weavers. Because of extremely low

parasitemias, identification of *Trypanosoma* spp. to species was not attempted.

The following birds were newly recognized as host species for some of the parasites studied: *Pycnonotus gracilis*, *H. otocompsae*, and *Leucocytozoon* sp.; *Prinia bairdii*, *H. wenyoni*; *Nectarinia olivacea*, *L. majoris*; *Euplectes afer*, *H. quelea*, and microfilariae of unidentified filariid nematode; *Euplectes macrourus*, *H. quelea*; *Ploceus aurantius*, *P. circumflexum*, and *P. vaughani*; *Ploceus nigerrimus*, *H. quelea*, and *L. majoris*; *Quelea erythrops*, *L. majoris*; *Estrilda astrild*, *H. orizivora*; *Estrilda atricapilla*, *H. orizivora*; *Hypargos nitidulus*, *H. orizivora*; *Pyre-*

TABLE II. Blood parasite prevalences in some African passeriform birds: results of selected studies (Palearctic migrants excluded).

Host family	Number infected/number examined (%) in:						
	Senegal (Bennett et al., 1978)	Cameroon (present study)	Chad (Williams et al., 1977)	Ethiopia (Ashford et al., 1976)	Uganda (Bennett et al., 1974, 1977)	Kenya (Peirce et al., 1977)	Zambia (Peirce 1984a)
Pycnonotidae	0/3 (0)	3/52 (6) H 2, L 4*	0/5 (0)	45/99 (45) H 20, L 20, P 1, T 5, M 10	26/375 (7) H 4, L 1, P 1, T 1, M 1	5/8 (63)	22/34 (65) H 44, L 47, P 6, T 6, M 15
Muscicapidae	0/4 (0)	3/58 (5) H 5	Not done	176/661 (27) H 14, L 5, P 6, T 3, M 5	14/132 (11) H 6, L 1, P 3, T 1	2/8 (25) H 29, P 13	40/107 (37) H 13, L 7, P 17, T 6, M 6
Nectarinidae	0/2 (0)	8/19 (42) H 16, L 11, P 16, T 16, M 5	1/4 (25) H 25	35/165 (21) H 19, L 2, P 1, T 1, M 2	9/105 (9) H 8, P 1	0/3 (0)	30/75 (40) H 28, L 9, P 4, T 8
Ploceidae	71/476 (15) H 12, P 2, T 1, M 1	22/68 (32) H 15, L 11, P 7, M 3	76/308 (25) H 15, P 3, T 1, M 8	255/806 (32) H 23, L 3, P 6, T 2, M 2	435/667 (65) H 64, L 3, P 1, T 1, M 2	41/65 (63) H 52, L 14, P 8, T 3	56/123 (46) H 35, L 11, P 8, T 1, M 1
Estrildidae	6/188 (3)† H 3, P 1, T 1	18/83 (22) H 22	3/24 (13) H 13, M 4	96/400 (24) H 18, L 1, P 2, T 3, M 4	6/41 (15) H 15	9/23 (39) H 26, L 4, T 4	67/236 (28) H 19, P 4, T 1, M 4
Totals (%)	77/673 (11)	52/240 (23)	80/341 (23)	607/2,131 (28)	490/1,320 (37)	57/107 (53)	215/575 (37)

\* Key: H = *Haemoproteus*; L = *Leucocytozoon*; P = *Plasmodium*; T = *Trypanosoma*; M = microfilariae of filariid nematodes. Percentage prevalence follows parasite genus.

† Consists entirely of *Lagonosticta senegalensis*.

*nestes ostrinus*, *H. orizivora*; and *Spermophaga haematina*, *H. orizivora*.

Among *Ploceus* spp., an association was noticed between nest dispersion and hematozoan fauna (Table I). Parasitism in *P. nigerrimus*, a species that habitually nests in large, tightly clustered colonies, was compared to that of *P. aurantius*, a species that nests either solitarily or in relatively dispersed colonies. Prevalence of infection with *H. quelea* was significantly higher in *P. nigerrimus* ( $P = 0.02$ , Fisher's exact test). Only 1 parasite species, *H. quelea*, was found to parasitize both host species, and again, its prevalence was significantly higher in *P. nigerrimus* ( $P = 0.02$ , Fisher's exact test).

## DISCUSSION

The haemoproteids of the Ploceidae have been considered variously to consist of several species or a single species (*H. passeris*) (Peirce, 1984c). *Haemoproteus* spp. are generally considered to be specific at the host familial level. In view of this and of the recent changes in avian classification (American Ornithologists' Union, 1983), we are tentatively regarding all of the haemoproteids of the Ploceidae to be *H. quelea* Marshall, 1912. It is possible that *H. quelea* and *H.*

*passeris* may prove to be synonymous (Peirce, 1984a).

We compared hematozoan prevalences in the 5 passerine families of our study with those of some other African studies (Table II). Overall hematozoan prevalences in African passerines appear to increase from west to east. This trend was particularly evident among the Pycnonotidae and the Muscicapidae. An exception was the relatively high prevalence of parasitism among the Nectarinidae at our study site compared with that in nectarinids studied in most other countries. The mix of species in each avian family differed among the localities surveyed, and the surveys were conducted at various times of the year. Infection with multiple genera of parasites was not common among birds from Cameroon, a result in accord with those of other African studies, but in contrast to North American birds (Greiner et al., 1975; Wink and Bennett, 1976; Bennett et al., 1978).

Differences observed in the hemoparasite fauna among the ploceids and the estrildids at our study site were similar to those reported in other studies conducted in neighboring Chad (Williams et al., 1977) and in Uganda (Bennett et al., 1974, 1977) (Table II). Among these 2 host fam-



ilies, Bennett et al. (1978) noted that the prevalence of parasitism in colonially nesting birds was higher than that in noncolonial nesters. Although sample sizes were small, we found a significant difference in the prevalence of *H. quelea* between the loosely colonial or solitary nester, *Ploceus aurantius* (5%), and the highly colonial nester, *P. nigerrimus* (44%). Collating comparable data on these birds from previous studies revealed that *P. nigerrimus* was indeed more likely to be infected with *H. quelea* than *P. aurantius* (67/93 [72%] vs. 12/73 [16%];  $P < 0.001$ , Fisher's exact test) (Peirce, 1969; Bennett et al., 1974, 1977; Wink and Bennett, 1976). The vectors of *Haemoproteus* spp. in Africa may be either midges (Ceratopogonidae) or louse flies (Hippoboscidae) (Fallis and Desser, 1977). Unlike ceratopogonids and common vectors of the other hematozoa (e.g., blackflies and mosquitoes), which tend to alight only for feeding, hippoboscid flies usually reside on the birds. Thus, highly colonial nesting behavior may facilitate transfer of infected hippoboscid flies, because hosts are in closer association than in loosely colonial or dispersed nests (hippoboscid flies were occasionally seen leaving various species of passerine birds during handling at our study site). Although this suggests that differences in prevalences of this and other hemoparasite genera among these hosts (Table I) may be related to host behavior, more birds need to be sampled during a given season at the same site to determine why such differences occur. Other possible explanations for the observed differences in hematozoan prevalences between these hosts include vector feeding preferences and variations in host susceptibility and resistance to the parasites.

In comparing results of various studies, it should be pointed out that local vector fauna and abundance as well as season may influence the results of avian hematozoa surveys (Bennett and Cameron, 1974; Atkinson et al., 1988). In tropical regions, seasonal variations in rainfall may explain observed temporal variations in hematozoan infection prevalences. Increased transmission of these parasites probably occurs during rainy seasons, when vectors are more abundant (Bennett et al., 1974). However, Peirce (1984e) found that relapses of parasitemias preceded rainy seasons in Zambia, suggesting hormonal or other influences on the parasites. In contrast, Bennett et al. (1978) found in Senegal that the highest recorded prevalences occurred in birds after the local rainy season. Sampling was conducted at

our study site just preceding and during the major rainy season. Longer term studies are required to determine whether seasonal fluctuations in hematozoan prevalence and intensity occur at our study site.

Another source of temporal variation in prevalence of avian hematozoans is the relative proportions of susceptible and immune hosts. Recently infected, parasite-naïve birds, mainly juveniles, likely would experience the highest parasitemias among the host population (e.g., Bennett, 1970; Seed and Manwell, 1977). Because parasites would be more obvious in blood smears from these birds, prevalences of hematozoans would appear highest during the breeding seasons, assuming that vectors are available then. Therefore, it will be important in future surveys to distinguish between juvenile and adult hosts. Conversely, during periods between relapses, birds with relatively low parasitemias would be more likely to be missed in surveys employing blood-smear examinations alone. This would tend to be reflected in relatively low prevalences. Thus, among adult birds, the use of more sensitive diagnostic tests for some of the parasites (e.g., blood culture for trypanosomes, iso-diagnosis for *Plasmodium* spp., and blood concentration using microhematocrit tubes for microfilariae and trypanosomes) would provide a better estimate of prevalence (Bennett, 1962; Kirkpatrick and Suthers, 1988).

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# **EIMERIA TRACHEMYDIS N. SP. (APICOMPLEXA: EIMERIIDAE) AND OTHER EIMERIANS FROM THE RED-EARED SLIDER, TRACHEMYS SCRIPTA ELEGANS (REPTILIA: TESTUDINES), IN NORTHCENTRAL TEXAS**

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**ABSTRACT:** *Eimeria trachemydis* n. sp. is described from the feces of 3/71 (4.2%) red-eared sliders, *Trachemys scripta elegans*, in Texas. Oocysts of the new species are ellipsoidal,  $25.0 \times 13.6$  (20.8–30.4  $\times$  12.0–16.0)  $\mu\text{m}$ , with a smooth, thin, apparently single-layered wall. A polar granule and oocyst residuum are present but a micropyle is absent. Sporocysts are elongate,  $14.4 \times 5.6$  (12.8–16.0  $\times$  5.0–6.4)  $\mu\text{m}$ , each with a Stieda body consisting of a slightly flattened region of the sporocyst wall bearing 2–5 stalked filaments; substieda body absent. Sporocyst residuum present, composed of a cluster of nonmembrane-bound granules. Sporozoites are elongate,  $12.2 \times 2.7$  (11.2–14.4  $\times$  2.4–3.2)  $\mu\text{m}$  *in situ*, each containing a spherical or ellipsoidal anterior refractile body and 1 (occasionally 2) midposterior spherical or ellipsoidal refractile bodies. A nucleus is located between the refractile bodies. In addition, prevalence data and specific identifications are presented on 10 other eimerians recovered from this host.

The red-eared slider, *Trachemys scripta elegans* (Wied), is one of the most common aquatic turtles in the midwestern and Mississippi River Valley regions of the United States, ranging from Indiana to New Mexico and south to the Gulf Coast into extreme northeastern Mexico (Conant, 1975). The species inhabits a wide variety of aquatic sites, but prefers quiet waters such as ponds, sloughs, and reservoirs containing muddy water and dense vegetation.

A plethora of information is available on various protozoan and metazoan parasites of *T. s. elegans* (see Ernst and Ernst, 1977, 1979, for reviews). However, little is known regarding the coccidia of this ubiquitous reptile. Sampson and Ernst (1969) described *Eimeria scriptae* from a single *Pseudemys* (= *Trachemys*, *sensu* Ward, 1984) *s. elegans*, from an unknown locality along the Mississippi River drainage of the United States, and Bone (1975) reported *E. pseudemydis* Lainson, 1968, from a specimen in Arkansas.

Between May 1987 and July 1988, we surveyed a large sample of various size- and age-classes of *T. s. elegans* for coccidian parasites and found several eimerians, including a previously undescribed species of *Eimeria*. Herein, we provide a description of the new species, along with prevalence data and specific identifications

of the other eimerians that we observed from this host.

## **MATERIALS AND METHODS**

Seventy-one juvenile and adult turtles (carapace length = 80–240 mm) were collected in cattle tanks or small lakes located in various localities of 5 counties of northcentral Texas with 2.5-cm-mesh wire hoop traps baited with sardines. Captured specimens were placed individually in 38-L glass aquaria and, soon after defecation (usually within 48 hr), were either retained for voucher specimens or returned to their original collection site. Fecal or intestinal samples from each turtle were placed in individual vials of 2.5% (w/v) aqueous  $\text{K}_2\text{Cr}_2\text{O}_7$  and stored at 4 C until examination. Samples were screened for the presence of coccidian oocysts by microscopy following flotation in modified Sheather's sugar solution (sp. gr. 1.30), and positive specimens were mailed to Kansas State University for further examination. Within 2 mo, sporulated oocysts were concentrated (as above) and examined and photographed with Nomarski interference-contrast optics. Oocysts were measured under an oil-immersion objective using an ocular micrometer. Measurements on 30 parasites are given as means (in  $\mu\text{m}$ ) followed by ranges in parentheses. Oocysts were 60 days old when measured.

## **RESULTS**

Forty-five of 71 (63.4%) turtles were infected with at least 1 of 11 eimerians (Table I). Of the infected animals, 15 (33.3%) harbored a single species, 14 (31.1%) had 2 species, 13 (28.9%) were infected with 3 species, 2 (4.4%) harbored 4 species, and 1 (2.2%) had 6 species. On the basis of overall prevalence, the 3 most common species were *Eimeria lutotestudinis* (28.2%), *E.*

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TABLE I. *Eimeria* spp. recovered from *Trachemys scripta elegans* in various counties of northcentral Texas.\*

<i>Eimeria</i> spp.	Texas county	Prevalence
<i>chrysemydis</i> Deeds and Jahn, 1939	Dallas	2/24 (8.3%)
	Johnson	1/32 (3.1%)
<i>graptemydos</i> Wacha and Christiansen, 1976	Dallas	5/24 (20.8%)
	Hood	3/13 (23.1%)
	Johnson	11/32 (34.4%)
<i>lutotestudinis</i> Wacha and Christiansen, 1976	Dallas	10/24 (41.7%)
	Denton	1/1 (100.0%)
	Johnson	9/32 (28.1%)
<i>marginata</i> (Deeds and Jahn, 1939) Pellérdy, 1974	Hood	1/13 (7.7%)
	Johnson	8/32 (25.0%)
<i>mitraria</i> (Laveran and Mesnil, 1902) Doflein, 1903	Coleman	1/1 (100.0%)
	Dallas	5/24 (20.8%)
	Denton	1/1 (100.0%)
	Hood	2/13 (15.4%)
	Johnson	9/32 (28.1%)
<i>pseudogeographica</i> Wacha and Christiansen, 1976	Dallas	4/24 (16.7%)
	Hood	1/13 (7.7%)
	Johnson	2/32 (6.3%)
<i>pseudemydis</i> Lainson, 1968	Dallas	7/24 (29.2%)
	Johnson	3/32 (9.4%)
<i>scriptae</i> Sampson and Ernst, 1969	Dallas	1/24 (4.2%)
	Johnson	1/32 (3.1%)
<i>tetradacrutata</i> Wacha and Christiansen, 1976	Hood	1/13 (7.7%)
<i>trachemydis</i> n. sp. McAllister and Upton, this paper	Dallas	3/24 (12.5%)
Unknown species	Johnson	1/32 (3.1%)

\* Counties with hosts not harboring a particular species not listed.

*graptemydos* (26.8%), and *E. mitraria* (25.4%). The least common eimerian was *E. tetradacrutata*, which was recovered from only 1 (1.4%) turtle. Oocysts of an unknown species of *Eimeria* resembling *E. mitraria* (Laveran and Mesnil, 1902) Doflein, 1903, were found in a single turtle from Johnson County. Sporulated oocysts were ovoid, measured  $16.5 \times 13.1$  ( $14.4\text{--}17.6 \times 12.0\text{--}14.4$ )  $\mu\text{m}$ , and had 2–7 conical (stylet-like) projections at either end of the oocyst, similar (but not identical) to the unnamed *Eimeria* sp. of Wacha and Christiansen (1976). Because only 1 turtle was found to be infected, we decline to name this species at this time until additional samples can be obtained. Three (4.2%) turtles were infected with the new species, which is described as follows.

#### DESCRIPTION

##### *Eimeria trachemydis* n. sp. (Figs. 1, 2)

Oocyst ellipsoid,  $25.0 \times 13.6$  ( $20.8\text{--}30.4 \times 12.0\text{--}16.0$ ), with a smooth, thin, apparently single-layered wall, ca. 0.6 thick; shape index 1.8 (1.5–2.2). A single ellipsoidal-spherical polar granule (1.6–1.8) is usually present at one pole, attached to inner surface of oocyst wall; micropyle absent. An oocyst residuum is present,  $6.8 \times 6.0$  ( $4.0\text{--}10.4 \times 3.2\text{--}9.6$ ) ( $n = 18$ ), consisting either of a loose cluster of granules or scattered

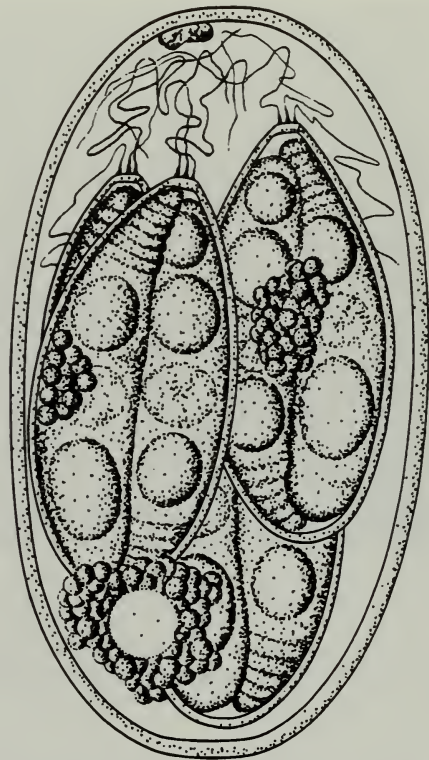


FIGURE 1. Line drawing of sporulated oocyst of *Eimeria trachemydis* n. sp. from the feces of *Trachemys scripta elegans*.

throughout the oocyst; intact residual granules usually enclose a large vacuolated area. Sporocysts elongate,  $14.4 \times 5.6$  ( $12.8\text{--}16.0 \times 5.0\text{--}6.4$ ), with a smooth, thin, single-layered wall, ca. 0.4 thick; shape index 2.6 (2.3–2.9). Stieda body present at one end of sporocyst, consisting of a slightly flattened region (ca. 1.6 wide) of the wall thickened to 0.6 with stalks bearing 2–5 filaments/sporocyst 6.0–10.0 long; substieda body absent. Sporocyst residuum present,  $4.0 \times 3.0$  ( $2.4\text{--}9.6 \times 1.8\text{--}4.0$ ) ( $n = 29$ ), in cluster, not membrane-bound. Sporozoites elongate,  $12.2 \times 2.7$  ( $11.2\text{--}14.4 \times 2.4\text{--}3.2$ ) *in situ*, arranged head-to-tail within the sporocyst. Each sporozoite contains a spherical or ellipsoidal anterior refractile body, 2.4 wide  $\times$  2.7 long ( $1.8\text{--}3.0 \times 2.4\text{--}3.2$ ), and a midposterior spherical refractile body, 2.5 wide  $\times$  3.5 long ( $2.0\text{--}3.0 \times 2.4\text{--}5.6$ ). A nucleus lies between the refractile bodies.

*Type host:* *Trachemys scripta elegans* (Wied, 1838) red-eared slider (Testudines: Emydidae). Voucher specimens are deposited in the Arkansas State University Museum of Zoology (ASUMZ 8475, 8493, 8553).

*Type specimens:* Syntypes (oocysts in 10% formalin) are deposited in the U.S. National Museum, Beltsville, Maryland 26705 as USNM 80459.

*Type locality:* U.S.A.: Texas: Dallas Co.: DeSoto, 1.6 km S I-20 on Bolton Boone Drive at Terrell Pond.





FIGURE 2. Nomarski interference-contrast photomicrograph of sporulated oocyst of *Eimeria trachemydis* n. sp. from the feces of *Trachemys scripta elegans*; fm, filament; ow, oocyst wall; pg, polar granule; rb, refractile body; sb, Stieda body; sr, sporocyst residuum; sw, sporocyst wall; sz, sporozoite. Bar = 5  $\mu$ m.

**Prevalence:** 3/71 (4.2%) of the turtles were infected; 0/1 (0%) Coleman County; 3/24 (12.5%) Dallas County; 0/1 (0%) Denton County; 0/13 (0%) Hood County; 0/32 (0%) Johnson County.

**Site of infection:** Unknown. Oocysts found in feces and intestinal contents.

**Sporulation:** Endogenous. Oocysts were passed fully sporulated.

**Etymology:** The specific epithet is derived from the generic name of the host.

## DISCUSSION

*Eimeria trachemydis* n. sp. most closely resembles *E. filamentifera* Wacha and Christiansen, 1979, from the common snapping turtle, *Chelydra serpentina serpentina* (Linnaeus), in Iowa, due to the presence of filaments at the end of the Stieda body. However, oocysts of *E. filamentifera* are wider and only slightly ovoid to ellipsoid, possess much shorter filaments, and lack a polar granule. The differences in these characteristics easily distinguishes the form we describe herein as new from *E. filamentifera*. In addition, the new species does not resemble any of the remaining 30+ eimerians from turtles (see Labbé, 1893; Simond, 1901; Laveran and Mesnil, 1902; Cerruti, 1930; Roudabush, 1937; Das Gupta, 1938; Deeds and Jahn, 1939; Carini, 1942; Chakravarty and Kar, 1943; Kar, 1944; Lainson, 1968; Ernst et al., 1969, 1971; Sampson and

Ernst, 1969; Ernst and Forrester, 1973; Wacha and Christiansen, 1974, 1976, 1977, 1979; Bone, 1975; Pluto and Rothenbacher, 1976; Ovez-mukhammedov, 1978).

In addition to being infected with the new species, all 3 turtles were multiply infected with other eimerians. These included: 2/3 (66.7%) with *E. lutotestudinis* Wacha and Christiansen, 1976, known previously from 4/26 (15.4%) Illinois mud turtles, *Kinosternon flavescens spooneri* Smith, in Iowa; 2/3 (66.7%) had *E. pseudogeographica* Wacha and Christiansen, 1976, known previously from 3/8 (37.5%) false map turtles, *Gratemys pseudogeographica* (Gray), and 5/22 (22.7%) western painted turtles, *Chrysemys picta bellii* (Gray), in Iowa; and 1/3 (33.3%) harbored *E. scriptae* Sampson and Ernst, 1969, known previously from 1/43 (2.3%) *T. s. elegans* from an unknown locality in the United States. These data combined with information provided in Table I suggest that little host specificity exists among certain turtle coccidia. However, in the absence of information on endogenous stages and cross-transmission studies of coccidia in turtles, we are left with the conclusion that oocysts are similar in more than one species of host.

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# COCCIDIA (APICOMPLEXA: EIMERIIDAE) FROM THE SUBTERRANEAN RODENT *CTENOMYS OPIMUS* WAGNER (CTENOMYIDAE) FROM BOLIVIA, SOUTH AMERICA

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**ABSTRACT:** Of 35 tuco-tucos (*Ctenomys opimus*) collected in Bolivia, South America, 31 (88%) had eimerian oocysts in their feces at the time they were examined. Eighteen (58%) of the 31 infected animals were concurrently infected with 2 or 3 eimerian species. Four species of *Eimeria* were recovered and are described as new species based on the characteristics of sporulated oocysts. Oocysts of *Eimeria granifera* n. sp. were ellipsoidal,  $21.1 \times 17.2$  ( $15\text{--}26 \times 11\text{--}20$ )  $\mu\text{m}$  with sporocysts ovoidal,  $11.3 \times 7.1$  ( $8\text{--}14 \times 5\text{--}9$ )  $\mu\text{m}$ . Oocysts of *Eimeria montuosi* n. sp. were spheroidal,  $24.2 \times 22.0$  ( $21\text{--}28 \times 18\text{--}25$ )  $\mu\text{m}$  with sporocysts ovoidal,  $10.5 \times 7.3$  ( $8\text{--}14 \times 6\text{--}9$ )  $\mu\text{m}$ . Oocysts of *Eimeria opimi* n. sp. were spheroidal to subspheroidal,  $24.3 \times 21.8$  ( $18\text{--}29 \times 15\text{--}26$ )  $\mu\text{m}$  with sporocysts ovoidal,  $11.6 \times 7.6$  ( $10\text{--}13 \times 6\text{--}9$ )  $\mu\text{m}$ . Oocysts of *Eimeria oruroensis* n. sp. were spheroidal to subspheroidal,  $27.3 \times 23.6$  ( $23\text{--}32 \times 20\text{--}28$ )  $\mu\text{m}$  with sporocysts ovoidal,  $13.2 \times 8.6$  ( $10\text{--}16 \times 8\text{--}11$ )  $\mu\text{m}$ .

The Ctenomyidae is 1 of 11 families belonging to the suborder Hystricognathi (see Mares and Ojeda, 1982). The geologic age of the family is Pliocene to Recent in South America (Nowack and Paradiso, 1983). Relatively little is known of the endoparasite fauna of these subterranean rodents, and, until the present paper, no coccidia had been reported from this group of mammals. Since 1984, fecal samples, helminths, ectoparasites, and other data (skin, skeleton, chromosomes, tissues for electrophoresis) have been collected from several populations of *Ctenomys opimus* Wagner from various localities in western Bolivia, South America (Fig. 1). In these hosts we found sporulated oocysts representing 4 new species of *Eimeria*, which we describe here.

## MATERIALS AND METHODS

All hosts were necropsied in the field promptly after capture. Organs were examined for the presence of metazoan parasites and fecal pellets were removed from the lower bowel of each animal and preserved in vials containing 2.0% aqueous (w/v)  $\text{K}_2\text{Cr}_2\text{O}_7$ . Upon return from Bolivia, samples were filtered and the fecal debris was then incubated at room temperature (24 C) for 20 days. Samples were then examined by coverslip flotation as described by Duszynski et al. (1982). Oocysts were measured with an ocular micrometer and photographed with Panatomic-X 35-mm film within a Zeiss Universal Photomicroscope equipped with both Neofluar and Nomarski-interference 100 $\times$  objective lenses. All measurements are in  $\mu\text{m}$  with the ranges in parentheses.

## RESULTS

The coccidians with which hosts were infected and collection localities are presented in Table I.

### *Eimeria granifera* n. sp.

(Figs. 2, 3, 11)

**Description:** Oocyst subspheroidal/ellipsoidal, wall of uneven thickness (Fig. 2)  $\sim 2.0$ , composed of 2 layers: outer wall smooth,  $\sim 3/4$  of total thickness, pale blue to transparent; inner layer yellow; polar body present in about 44% of the oocysts; oocyst residuum absent; sporulated oocysts ( $n = 75$ )  $21.1 \times 17.2$  ( $15\text{--}26 \times 11\text{--}20$ ) with L:W ratio 1.2 ( $1.1 \times 1.9$ ); sporocysts ( $n = 75$ ) ovoidal,  $11.3 \times 7.1$  ( $8\text{--}14 \times 5\text{--}9$ ) with L:W ratio 1.6 ( $1.2 \times 2.0$ ); button-like Stieda body present (Figs. 2, 3), but sub- and parastieda bodies absent; sporocyst residuum of 10–12 large globules in a compact mass of varying shape, wedged between sporozoites (Figs. 2, 3); each sporozoite large, folded, highly granular with a large posterior refractile body (Figs. 3, 11). Oocysts were 274–275 days old when measured.

### Taxonomic summary

**Diagnosis:** Oocysts of this eimerian do not resemble those from any eimerian previously described from New World hystricognath rodents.

**Type host:** *Ctenomys opimus* Wagner, Museum of Southwestern Biology, Division of Mammalogy, MSB 57200, NK 14776 (female), N. Olds #980, 3 Oct 1986.

**Type locality:** 3 km W of Huancaroma, Rio Desaguadero, 3,720 m, Department of Oruro, Bolivia, South America ( $17^{\circ}40'S$ ,  $61^{\circ}31'W$ ).

**Prevalence:** Found in 23 of 35 (66%) *C. opimus*.

**Site of infection:** Unknown, oocysts recovered from feces.

**Material deposited:** Syntypes (=phototypes, see Bandoni and Duszynski, 1988) of sporulated oocysts, USNM Helm. Coll. No. 80445.

**Etymology:** The *nomen triviale* is derived from the word *granifer* (L., grain-carrying); this is descriptive of the highly granular sporozoites.

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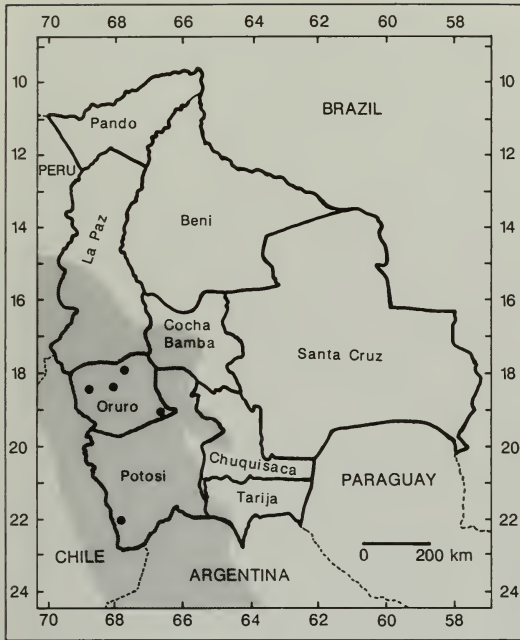


FIGURE 1. Map of Bolivia, South America, showing its contiguous neighbors and the arrangement of its Departments (states). Shaded area shows the distribution of *Ctenomys opimus*. Dots represent collection localities.

#### *Eimeria montuosi* n. sp. (Figs. 4, 12)

**Description:** Oocysts spheroidal or nearly so with thick wall ~3.0, consisting of 2 or 3 layers: outer layer having large protruding bumps on the surface, yellow to gold, ~ $\frac{1}{4}$  of the total thickness (Fig. 4, insert); oocyst residuum a compact mass of large granules, ~6; polar body absent; sporulated oocysts ( $n = 42$ )  $24.2 \times 22.0$  ( $21-28 \times 18-25$ ) with L:W ratio 1.1 ( $1.0 \times 1.2$ ); sporocysts ( $n = 42$ ) ovoidal,  $10.5 \times 7.3$  ( $8-14 \times 6-9$ ) with L:W ratio 1.4 ( $1.2 \times 1.9$ ); sporocysts with small Stieda body, but sub- and parastieda bodies absent; sporocyst residuum consisting of 6 or more small globules separating the sporozoites. Oocysts were 298–330 days old when measured.

#### Taxonomic summary

**Diagnosis:** Oocysts of this eimerian do not resemble those from any eimerian previously described from New World hystricognath rodents.

**Type host:** *Ctenomys opimus* Wagner, Museum of Southwestern Biology, Division of Mammalogy, MSB 57202, NK 14559 (female), N. Olds #861, 12 Sep 1986.

**Type locality:** 5 km W, 1 km N Pomata Ayte, Rio Barros, Department of Oruro, Bolivia, South America ( $18^{\circ}40'S$ ,  $67^{\circ}59'W$ ).

**Prevalence:** Found in 1 of 35 (3%) *C. opimus*.

**Site of infection:** Unknown, oocysts recovered from feces.

**Material deposited:** Syntypes (phototypes) of sporulated oocysts, USNM Helm. Coll. No. 80446.

TABLE I. *Eimeria* spp. recovered from 35 *Ctenomys opimus* from the western high altitude of Bolivia, South America.

Department: Location	No. hosts infected/ examined (%)	<i>Eimeria</i> spp.
<b>Oruro:</b>		
Cruce Ventilla	5/6 (83)	<i>opimi</i> , <i>granifera</i>
Estancia Agua Rica	0/1 (0)	—
Huancaroma	18/19 (95)	<i>opimi</i> , <i>granifera</i> , <i>oruroensis</i>
Pomata Ayte	4/5 (80)	<i>opimi</i> , <i>granifera</i> , <i>montuosi</i>
<b>Potosi:</b>		
La Laguna	4/4 (100)	<i>opimi</i> , <i>granifera</i>
Totals (5 locations)	31/35 (88)	4

**Etymology:** The *nomen triviale* is derived from the word *montuosus* (L., mountainous); this is descriptive of the large protruding bumps on the surface of the oocyst wall.

#### *Eimeria opimi* n. sp. (Figs. 5–7, 13)

**Description:** Oocyst spheroidal to subspheroidal, wall ~1.5, composed of at least 2 layers: outer layer finely sculptured (Fig. 7, insert), ~ $\frac{3}{4}$  of total thickness, colorless to pale blue; inner layer smooth, transparent to pale blue; dumbbell-shaped polar body present (Fig. 5); oocyst residuum (~5.3) composed of 8–10 uniform granules in a compact mass (Fig. 6); sporulated oocysts ( $n = 55$ )  $24.3 \times 21.8$  ( $18-29 \times 15-26$ ) with L:W ratio 1.1 ( $1.0 \times 1.7$ ); sporocysts ( $n = 55$ ) ovoidal,  $11.6 \times 7.6$  ( $10-13 \times 6-9$ ) with L:W ratio 1.5 ( $1.3 \times 1.9$ ); nipple-like Stieda body present (Figs. 5–7), but sub- and parastieda bodies absent; sporocyst residuum of 2–3 granules (Fig. 7) separating the sporozoites; each sporozoite transparent with large posterior refractile body (Fig. 7). Oocysts were 1,024–1,025 days old when measured.

#### Taxonomic summary

**Diagnosis:** Oocysts of this eimerian do not resemble those from any species previously described from New World hystricognath rodents.

**Type host:** *Ctenomys opimus* Wagner, Museum of Southwestern Biology, Division of Mammalogy, MSB 55372, NK 11564 (male), J. A. Cook #1264, 6 Aug 1984.

**Type locality:** 2.5 km NE of Huancaroma, Department of Oruro, Bolivia, South America ( $17^{\circ}40'S$ ,  $67^{\circ}27'W$ ).

**Prevalence:** Found in 28 of 35 (80%) *C. opimus*.

**Site of infection:** Unknown, oocysts recovered from feces.

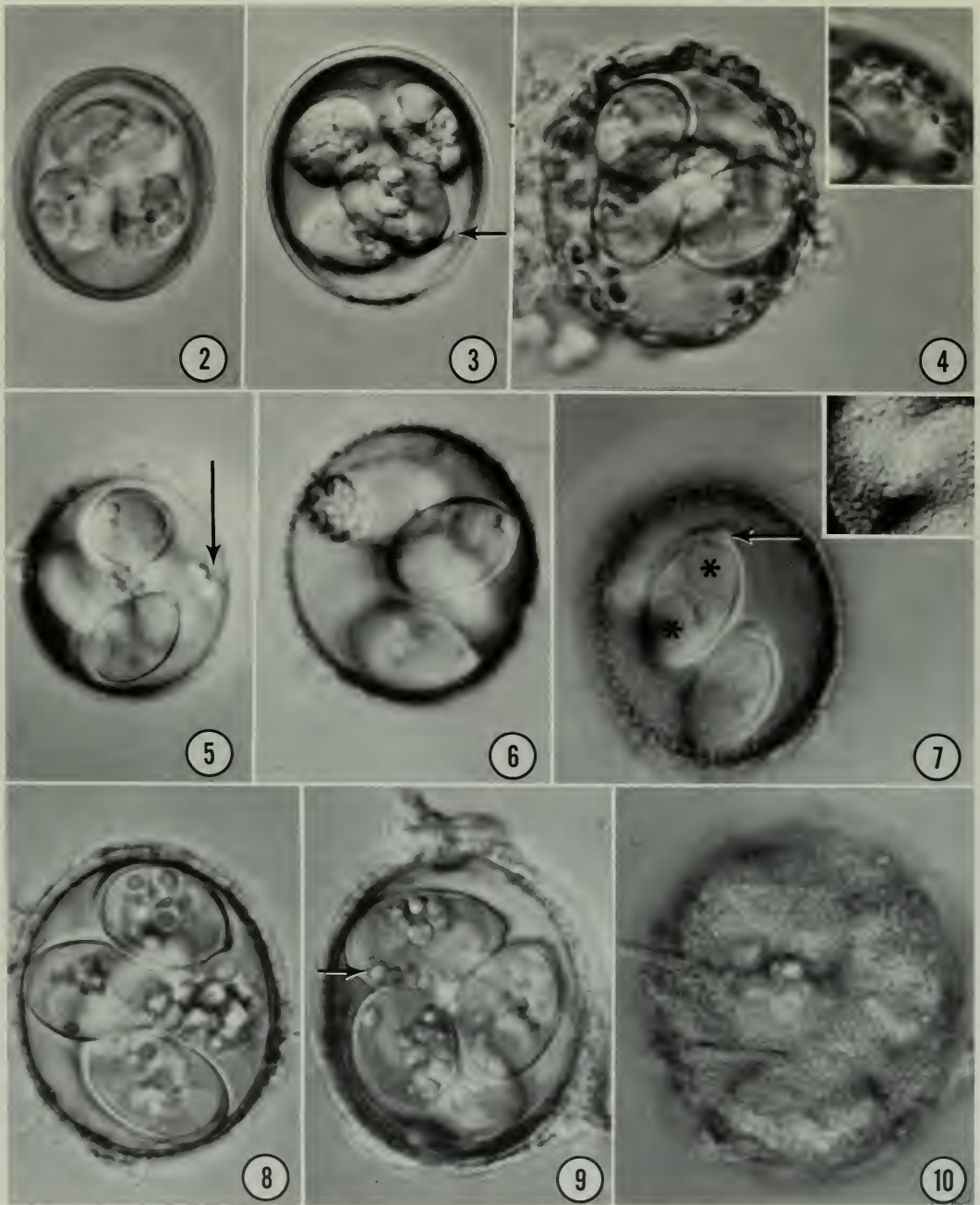
**Material deposited:** Syntypes (phototypes) of sporulated oocysts, USNM Helm. Coll. No. 80447.

**Etymology:** The *nomen triviale* is derived from the specific name of the host.

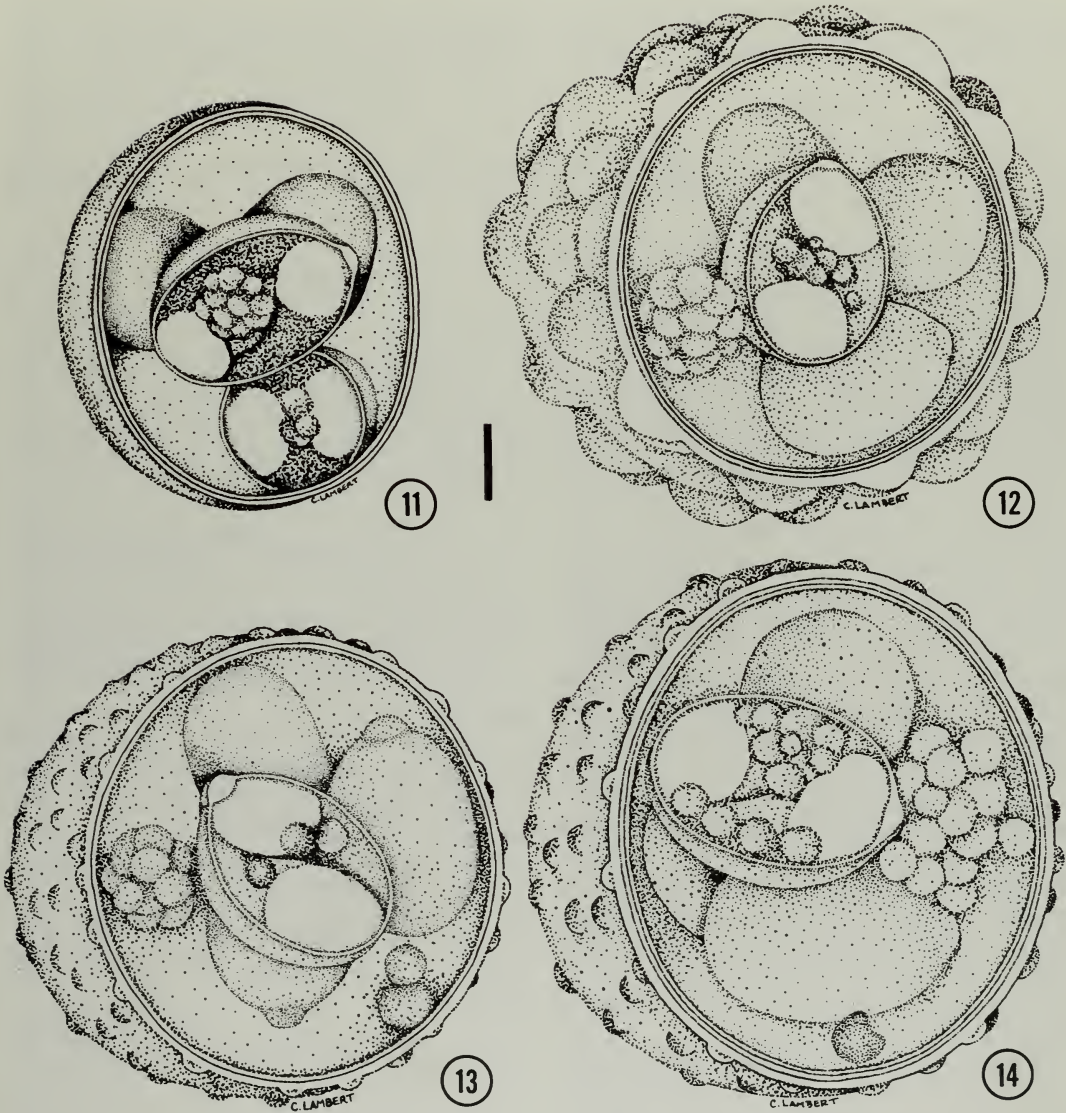
#### *Eimeria oruroensis* n. sp. (Figs. 8–10, 14)

**Description:** Oocyst spheroidal to subspheroidal, wall 2.3–3.0, composed of at least 3 layers: outer wall rough





FIGURES 2-10. Photomicrographs of sporulated oocysts of coccidia recovered from the feces of *Ctenomys opimus*.  $\times 1,860$ . 2, 3. *Eimeria granifera* n. sp. Note button-like Stieda body (3, arrow), sporocysts residuum (2), and large, folded, highly granular sporozoites with large posterior refractile body. 4. *Eimeria montuosi* n. sp. Note large, protruding bumps on surface of oocyst. 5-7. *Eimeria opimi* n. sp. 5. Note dumbbell-shaped polar body (arrow). 6. Sporulated oocyst showing compact oocyst residuum. 7. Sporocysts with nipple-like Stieda body (arrow), large posterior refractile body (\*), and finely sculptured nature of oocyst wall (insert). 8-10. *Eimeria oruroensis* n. sp. 8. Sporulated oocyst showing oocyst residuum in a loosely packed mass. 9. Sporocyst residuum forming a line along sporocyst wall (arrow), and separating the 2 sporozoites. 10. Sculptured nature of oocyst wall.



FIGURES 11–14. Line drawings of sporulated oocysts of coccidia recovered from the feces of *Ctenomys opimus*; bar = 3  $\mu$ m. 11. *Eimeria granifera*. 12. *Eimeria montuosi*. 13. *Eimeria opimi*. 14. *Eimeria oruroensis*.

(Fig. 10),  $\sim 3/4$  of total thickness, yellow to gold, always with adherent debris; middle layer pale yellow in color; innermost layer dark brown; polar body present; oocyst residuum ( $\sim 9$ ) composed of 10–15 globules of varying size in a loosely packed mass (Fig. 8); sporulated oocysts ( $n = 50$ )  $27.3 \times 23.6$  ( $23\text{--}32 \times 20\text{--}28$ ) with L:W ratio 1.2 ( $1.0 \times 1.7$ ); sporocysts ( $n = 50$ ) ovoidal,  $13.2 \times 8.6$  ( $10\text{--}16 \times 8\text{--}11$ ) with L:W ratio 1.5 ( $1.2 \times 1.8$ ); Stieda body present, but sub- and parastieda bodies absent; sporocyst residuum consists of large globules of varying shape and size separating the 2 sporozoites or forming a line along the sides of the sporocyst wall (Figs. 8, 9); each sporozoite with a posterior refractile body. Oocysts were 1,023–1,035 days old when measured.

#### Taxonomic summary

**Diagnosis:** Oocysts of this eimerian do not resemble those from any species previously described from New World hystricognath rodents.

**Type host:** *Ctenomys opimus* Wagner, American Museum of Natural History, Division of Mammalogy, AMNH 260840, NK 11514 (male), S. Anderson #7890, 4 Aug 1984.

**Type locality:** 3.5 km E of Huancaroma, Department of Oruro, Bolivia, South America ( $17^{\circ}40'S$ ,  $67^{\circ}27'W$ ).

**Prevalence:** Found in 1 of 35 (3%) *C. opimus*.

**Site of infection:** Unknown, oocysts found in feces.

**Material deposited:** Syntypes (phototypes) of sporulated oocysts, USNM Helm. Coll. No. 80444.



*Etymology:* The *nomen triviale* combines the name of the department in Bolivia in which the infected host was caught and *-ensis* (L., belonging to).

### DISCUSSION

The genus *Ctenomys* Blainville includes approximately 33 currently recognized species (Honacki et al., 1982). These rodents occur in suitable habitats from about 10°S latitude southward to the Strait of Magellan. *Ctenomys opimus* has one of the most extensive geographic distributions compared to other members of the genus (Fig. 1). Colonies occur at altitudes up to >4,000 m in the altiplano region of Bolivia and in habitats characterized as Puna and high altitude desert of Peru, Bolivia, Chile, and Argentina (Gallardo, 1979; Mares and Ojeda, 1982). *Ctenomys opimus* is a strictly subterranean species with populations occurring in small isolated areas throughout their range in Bolivia (Gardner, pers. obs.). These rodents display some of the same ecological and morphological attributes as other subterranean rodents (Nevo, 1979). For example, members of the genus *Thomomys* Weid-Neuwied in the Nearctic exhibit low vagility, relatively low density, and patchy distributions (Patton, 1972).

Up to the present, the coccidia of *Ctenomys* have not been studied, and the number of species that exist of both host and parasite and their phylogenetic and coevolutionary relationships are unknown. Pellérdy (1974) listed 23 described species of *Eimeria* from hystricognath rodents; however, none of those listed are from the host *Ctenomys*, nor do they resemble the eimerians described in the present paper.

At present we are analyzing the coccidians from 9 other species of *Ctenomys*. In subsequent papers, we hope to discuss the patterns and processes of speciation, diversification, and coevolution among members of the genus *Ctenomys* and their parasites.

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## HISTOCHEMICAL LOCALIZATION OF ACETYLCHOLINESTERASE IN THE CEREBRAL GANGLIA OF *FASCIOLA HEPATICA*, A PARASITIC FLATWORM

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**ABSTRACT:** Acetylcholinesterase activity was found in the cell bodies and extracellularly in the neuropile of the cerebral ganglia of the adult trematode parasite, *Fasciola hepatica*. Within neuronal cell bodies of the cerebral ganglion, acetylcholinesterase reaction product was found in the endoplasmic reticulum, in the cisternae of the Golgi apparatus, and in secretory vesicles near the inner (releasing face) cisternae. Acetylcholinesterase reaction product was not seen intracellularly within any nerve processes. The reaction product was found around the somatic cell membranes and in the extracellular space between closely apposed nerve processes in the neuropile. Acetylcholinesterase reaction product was associated with synaptic endings that contained clear spheroidal synaptic vesicles, and the reaction product was localized at the site of synaptic contact between the zone of apposition of the pre- and postsynaptic terminals. This intracellular and extracellular distribution of the enzyme is consistent with its function as the degrading enzyme in cholinergic transmission.

*Fasciola hepatica* is a parasite that belongs to the class Trematoda in the phylum Platyhelminthes. The classification of the Platyhelminthes is currently in a state of flux (Ehlers, 1985). Traditionally, it is thought that the phylum embraces 4 classes, 3 of which are entirely parasitic (Barnes, 1987). The fourth class, Turbellaria, is free-living. Of all the bilateral phyla, the flatworms have long been considered the most primitive and it is in these animals that we see the first appearance of centralized "brains" or cerebral ganglia. Morphological studies show a wide range of variation in the development and complexity of the nervous system in this phylum. *Convoluta* spp. (Turbellaria) possess a general superficial plexus with indistinct nerve cords and irregular commissures, whereas *Notoplana* spp. (Turbellaria) and *Fasciola hepatica* (Trematoda) have highly organized multilobed brains with 2 pairs of prominent longitudinal nerve cords (Bullock and Horridge, 1965). It is believed that the steps in the evolution of the nervous system from simple nerve nets to features common to the CNS of higher invertebrates and vertebrates may all be found in animals of this phylum (Lentz, 1968; Koopowitz and Keenan, 1982). Although many investigators have worked on various aspects of the neurobiology of these animals, our understanding is still limited (Bullock, 1984). For example, the kinds of neurotransmitters and the mechanisms of neurotransmission used in these primitive nervous systems are still a mystery al-

though several putative neurotransmitter substances have been identified (Hillman et al., 1976; Bennett and Gianutsos, 1977; Keenan and Koopowitz, 1982; Hauser and Koopowitz, 1987; Ribeiro and Webb, 1987). Studies on the behaviour and neurobiology of flatworms increasingly suggest that "primitiveness does not equate with simplicity" and the mechanisms of neural transmission used by these worms may be comparable with those of higher phyla (Koopowitz and Keenan, 1982).

In *Fasciola hepatica*, it has been demonstrated clearly that acetylcholine (ACh) has an inhibitory effect on muscle contractions (Chance and Mansour, 1953; Sukhdeo et al., 1986), that the ACh receptors are primitive in their pharmacological types (i.e., mixed nicotinic and muscarinic properties) (Sukhdeo et al., 1986), and that the worms possess the biochemical machinery to synthesize and degrade endogenous ACh (Sukhdeo et al., 1986). Although these data constitute strong evidence that ACh is a neurotransmitter in this parasite, they are not conclusive. The identification of neurotransmitters requires the additional demonstration that the neurotransmitter compound is located in appropriate sites in the nervous system (Gainer and Brownstein, 1981; Shepherd, 1983). Acetylcholinesterase (AChE; E.C. 3.1.1.7), the enzyme that degrades ACh, can be localized histochemically. This method can be used to indirectly identify the functional localization of ACh (Lewis and Shute, 1966), although it is possible for AChE to be associated with noncholinergic neurons and nerve processes (Bradford, 1986). The object of this study was

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to determine the localization of AChE in the nervous system of *F. hepatica*.

## MATERIALS AND METHODS

Adult *Fasciola hepatica* were collected from the bile ducts of Wistar white rats (Woodlyn Labs, Guelph, Ontario) infected 3–4 mo previously. The worms were kept in Hedon-Fleig buffered ringer solution (Sukhdeo et al., 1986) until they were used (less than 5 min). The head region of the fluke, approximately 2 mm from the anterior tip, was removed and fixed for 90 min at room temperature. The fixative consisted of 0.5% glutaraldehyde and 4% formaldehyde in 0.1 M sodium cacodylate solution (pH 7.4).

After fixation, the tissue samples were passed through a graded series of glycerol (cryoprotectant) (10%, 20%, and 30%) for 7 min each. The samples were immersed in a slurry of Freon (fluorocarbon-12 (dichlorodifluoromethane; HistoFreeze, Fisher Scientific, Toronto, Ontario) for 15 sec and in liquid nitrogen for 10 sec before being embedded into a water-soluble compound for cryostat sectioning (Tissue-Tek, Canlab, Toronto, Ontario). Thick cryostat (Ames Lab-Tek Cryostat) sections (40–60  $\mu$ m) were treated for the histochemical localization of AChE.

The procedure for the localization of acetylcholinesterase at the electron microscopic level was modified from the methods of Kása and Csillik (1966) and Jensen et al. (1978). The cryostat sections were washed in several changes of a 0.1 M sodium cacodylate solution containing 0.1 M sucrose and 0.05%  $\text{CaCl}_2$  (pH 7.4) for 90 min. Following the last wash, the tissue sections were preincubated for 40 min in either  $10^{-4}$  M ethopropazine hydrochloride (Sigma Chemical Co., St. Louis, Missouri), a nonspecific cholinesterase inhibitor, or  $10^{-4}$  M BW284C51 (1,5-bis-[4-allyldimethylammonium phenyl]-pentan-3,1-dibromide) (Sigma Chemical Co.), a specific inhibitor of acetylcholinesterase activity (Jessen et al., 1978). The sections were then incubated for 90 min in the enzyme reaction solution (Kása and Csillik, 1966) that contained the same inhibitors as the preincubation solution. Two brief rinses in the wash solution were then followed by 7 min in a 25% sodium sulfide solution. The sections were again rinsed twice in wash solution and postfixed in 1%  $\text{OsO}_4$  in Millonig buffer (pH 7.4) for 1 hr. Some sections were stained en bloc in 0.5% uranyl acetate in an acetate buffer (pH 5.4) for 2 hr prior to dehydration. After dehydration with alcohol, the sections were left overnight in a propylene oxide: Epon-Araldite (50:50) mixture and embedded in Epon-Araldite the following day. Gold serial sections were cut with a diamond knife on a Sorval UM 2 ultramicrotome and placed on single-slot copper grids coated with formvar. Some of the sections were postfixed with lead citrate to produce

some contrast and structural details. A Philips 201-C electron microscope was used for viewing and photography.

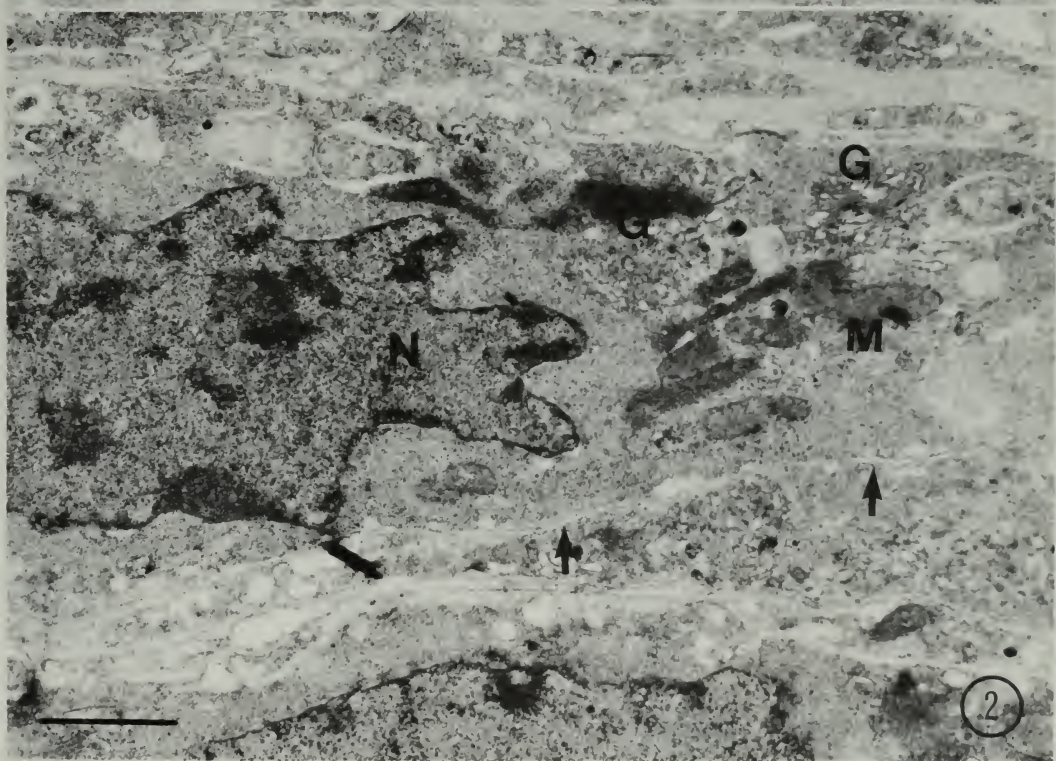
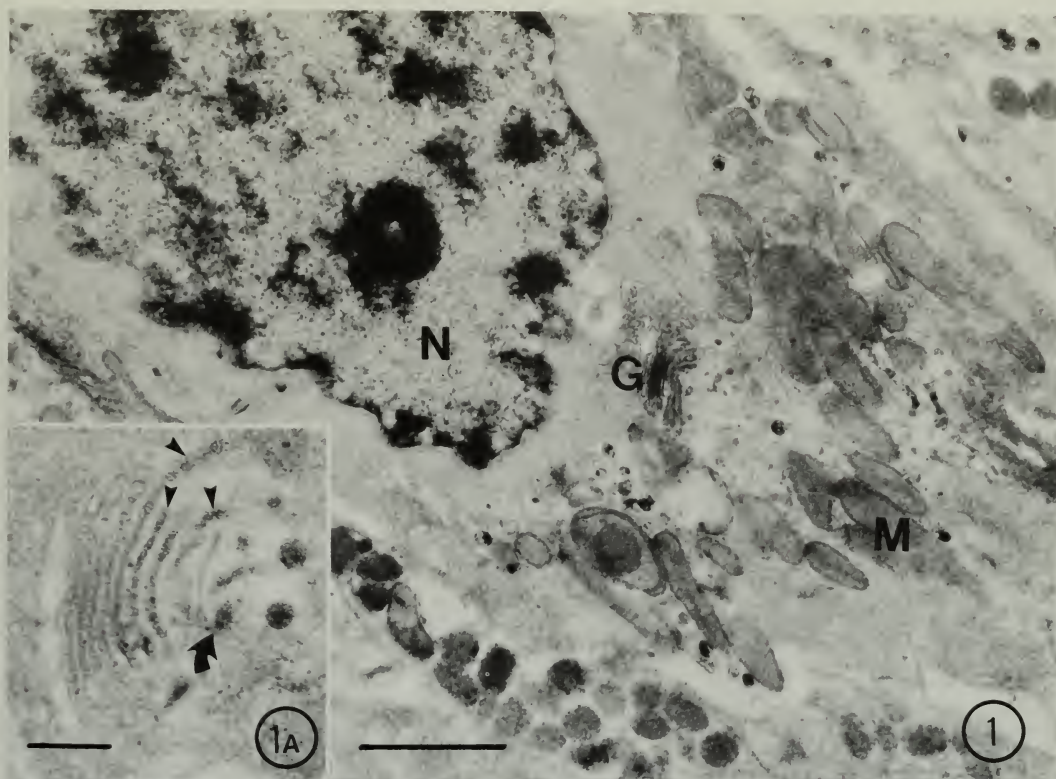
## RESULTS

*Fasciola hepatica* has 2 large cerebral ganglia that contain dispersed neuropiles with scattered neuron cell bodies that are not organized in a cell rind. In the cerebral ganglia, AChE activity was found intracellularly in the soma of neurons and extracellularly on the surface membranes. The specificity of the AChE localization was tested by incubating tissues in the presence of the specific AChE inhibitor, BW284C51. There was almost complete absence of reaction product in these sections (see Figs. 2, 7). All other tissues were incubated with the nonspecific cholinesterase inhibitor, ethopropazine (Figs. 1, 3–6, 8–11).

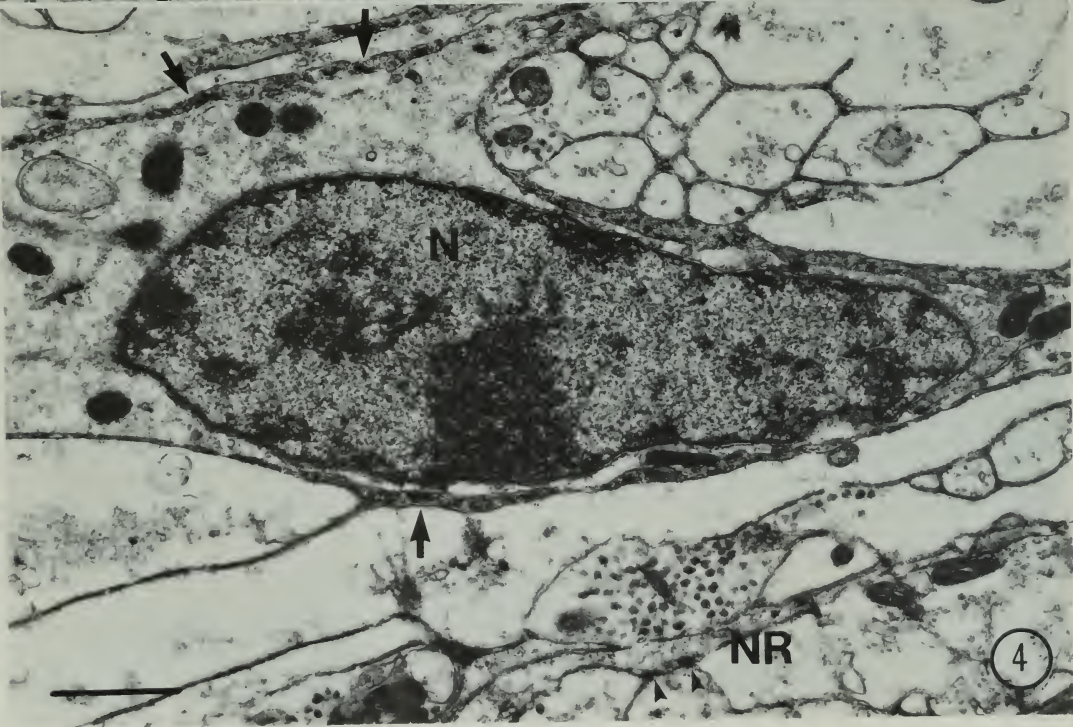
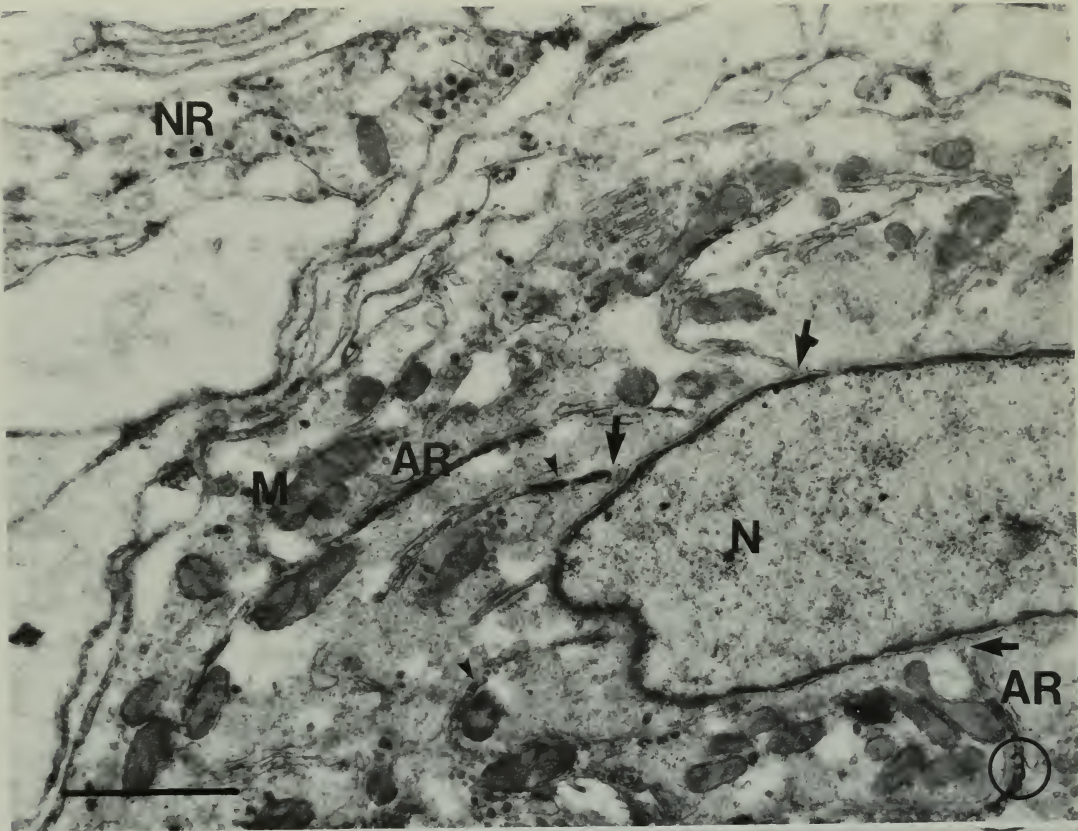
Intracellularly, the AChE reaction product was found predominantly in the cisternae of the Golgi apparatus (Fig. 1), and was usually seen in the cisternae toward the inner (releasing) face where the secretory vesicles bud off (Fig. 1 inset). Controls incubated with BW284C51 show no reaction product in the cisternae of the Golgi apparatus (Fig. 2). The AChE reaction product was also found in the cisternae of the agranular reticulum (AR) of these neurons (Fig. 3). In some cases, the AR appears closely apposed to the nuclear envelope although no reaction product was observed to stain the nuclear envelope (Fig. 3).

The AChE reaction product was also associated with the outer surface membranes (extracellular) of the soma of neurons and of nerve processes in the neuropile (Figs. 3–6). In Figures 3 and 4, the reaction product is seen extracellularly to the somatic surface of neurons that are located next to the neuropile. The majority of the extracellularly located AChE reaction product was found between nerve processes in the neuropile (Figs. 5, 6). All nerve processes are unmyelinated and are closely apposed to each other in the neuropile. The reaction product was associated with synaptic endings (Figs. 8–10) but sometimes occurred as discrete patches in the extracellular

FIGURES 1, 2. 1. Soma of a neuron in the cerebral ganglion. AChE reaction product is found in the cisternae of the Golgi complex (G). The nucleus (N) and the nuclear membrane are not stained. The inset (1A) shows the reaction product in the cisternae (arrowheads) toward the inner face of the Golgi and in the vesicles that bud off (curved arrows) from the membranes. M, mitochondria. Scale bar = 1  $\mu$ m; inset scale bar = 0.25  $\mu$ m. 2. Tissue section that was treated with BW284C51, the AChE inhibitor. Soma of a neuron similar to Figure 1 except the Golgi complexes (G) are unstained. Reaction product is also not found on the extracellular surface of the somatic cell membranes (arrows). N, nucleus; M, mitochondria. Scale bar = 1  $\mu$ m.









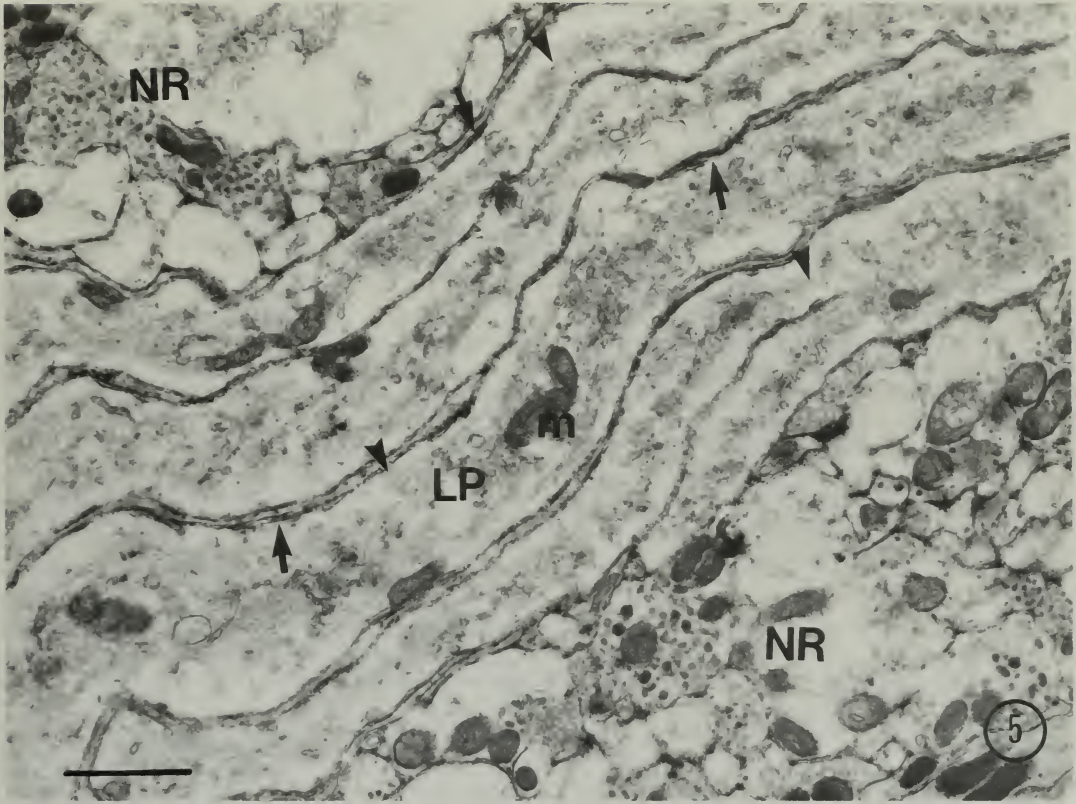


FIGURE 5. Section through the neuropile showing longitudinal profiles of nerve processes (LP) and cross-sectional profiles of processes in the neuropile (NR). AChE reaction product is found extracellularly between the nerve processes (arrows). Arrowheads, microtubules; M, mitochondria. Scale bar = 1  $\mu$ m.

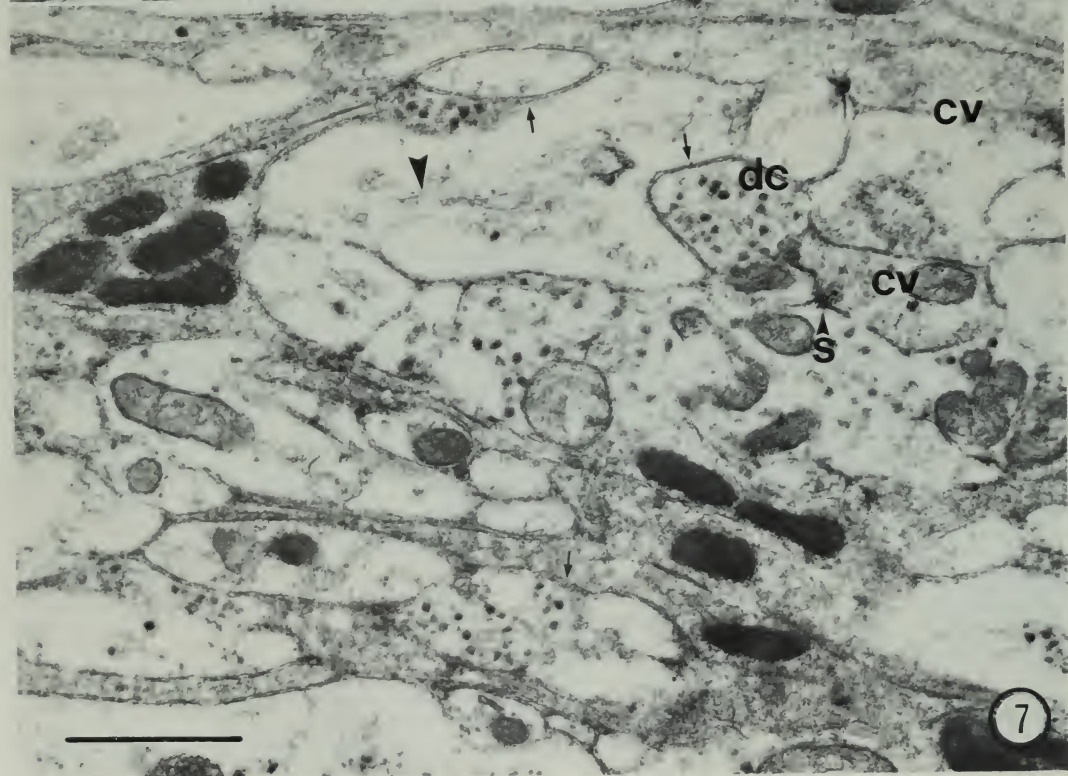
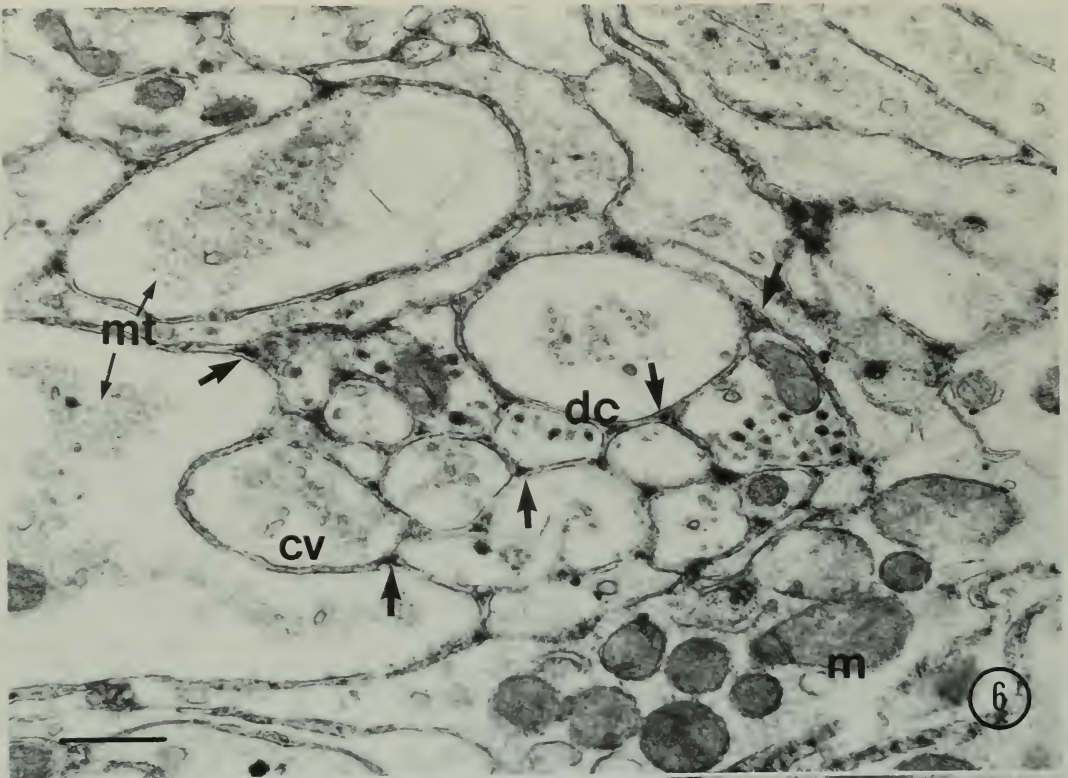
spaces in areas of the neuropile with no synaptic endings (Figs. 5, 6). There appears to be no correlation between the presence of enzyme reaction product in the extracellular space around nerve processes and the type of vesicles found in those nerve processes (Fig. 6). In contrast, in a similar cross section of the neuropile that was incubated with BW284C51, there was a complete absence of AChE reaction product from the extracellular space (Fig. 7).

At synapses, the AChE reaction product was associated with only 1 morphological type of synaptic ending that was characterized by the presence of clear spheroidal synaptic vesicles (30–40 nm) (Figs. 8–10). The enzyme reaction product

was found only in the synaptic contact zone between the presynaptic membrane density and cluster of clear spheroidal synaptic vesicles, and the postsynaptic density (Figs. 8–10). No enzyme reaction product was found outside the immediate vicinity of the synaptic contact zone. A large number of triad- or wedge-type synapses were observed in the neuropile with AChE reaction product in the extracellular cleft (Figs. 9, 10). These synaptic endings also contained clear spheroidal synaptic vesicles. However, it is important to note that not all synaptic endings that contained the clear spheroidal synaptic vesicles demonstrated AChE reaction product in their synaptic clefts (Fig. 11).

FIGURES 3, 4. 3. Soma of a neuron with the agranular reticulum (AR) stained with AChE reaction product. The AR is occasionally seen closely apposed to the unstained nuclear membrane (arrows) and mitochondria (M). The reaction product seems to appear as discrete globules in the AR (arrowheads). N, nucleus. Scale bar = 1  $\mu$ m. 4. A neuronal soma with AChE reaction product in the extracellular space around the cell membrane (arrows) and around processes (arrowheads) in the neuropile (NR). Scale bar = 1  $\mu$ m.





## DISCUSSION

Acetylcholinesterase is the enzyme in the synaptic cleft that hydrolyzes acetylcholine released at cholinergic synapses. The enzyme is a glycoprotein and in most vertebrate central and peripheral nervous systems that have been studied, it is synthesized in the endoplasmic reticulum, packaged in the Golgi apparatus of the neuronal cell body (Droz et al., 1979; Rotundo, 1984), and carried by the fast axonal transport system to its ultimate destination in the extracellular space (Rotundo and Fambrough, 1980; Hammerschlag and Stone, 1982). In the neurons of *F. hepatica*, the AChE reaction product was localized in the agranular endoplasmic reticulum, the cisternae of the Golgi apparatus, and in secretory vesicles from the Golgi. This localization pattern represents the intermediary process in the synthesis, packaging, and transport along the axon (Gisiger et al., 1975; Hammerschlag and Stone, 1982). Similar localization in identified cholinergic neuron cell bodies have been reported in vertebrates (Kása and Csillik, 1966; Jessen et al., 1978; Spencer and Baker, 1986). The Golgi-derived secretory vesicles are believed to transform into vesiculotubular structures that act as transport vectors within the axons (Rotundo and Fambrough, 1980; Hammerschlag and Stone, 1982) and axonal tubules stained with AChE reaction product have been reported (Kása, 1968). However, evidence of vesicle-mediated transit of AChE was not observed in *F. hepatica*.

The extracellular localization of AChE is associated with the somatic and nerve process membranes in the neuropile. This pattern of extrasynaptic extracellular localization of AChE activity has been observed in both vertebrates (Kása and Csillik, 1966; Jessen et al., 1978; Spencer and Baker, 1986) and higher invertebrates (Smith and Treherne, 1965; Newman et al., 1968; Silver, 1972). Acetylcholinesterase reaction product was associated with only 1 morphological type of synaptic endings. These synaptic endings were characterized by accumulation of clear

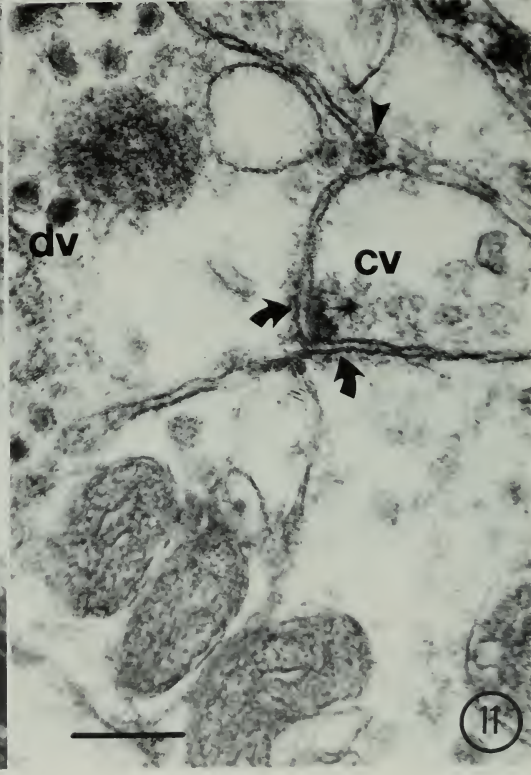
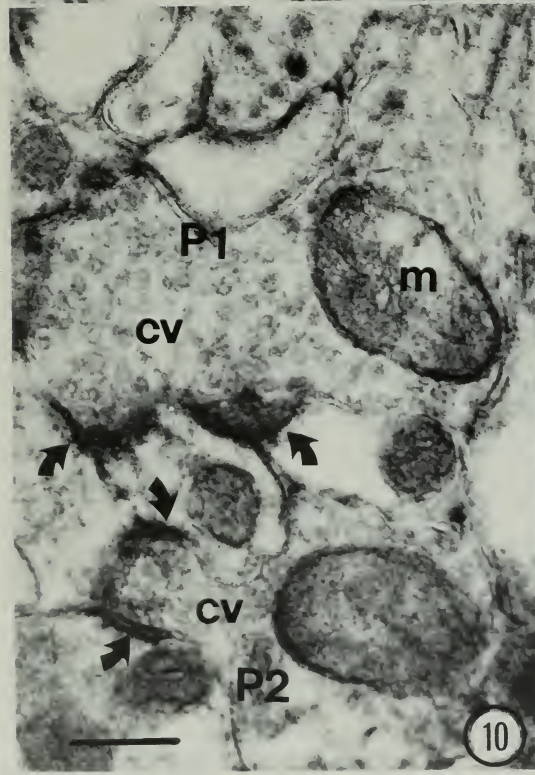
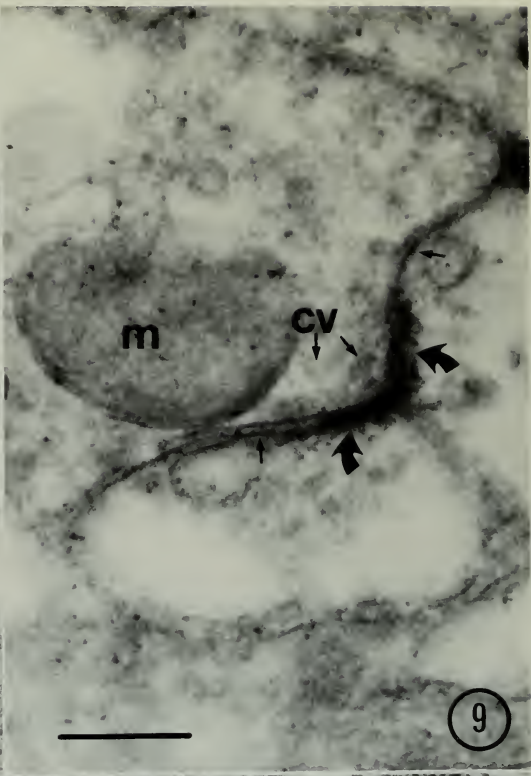
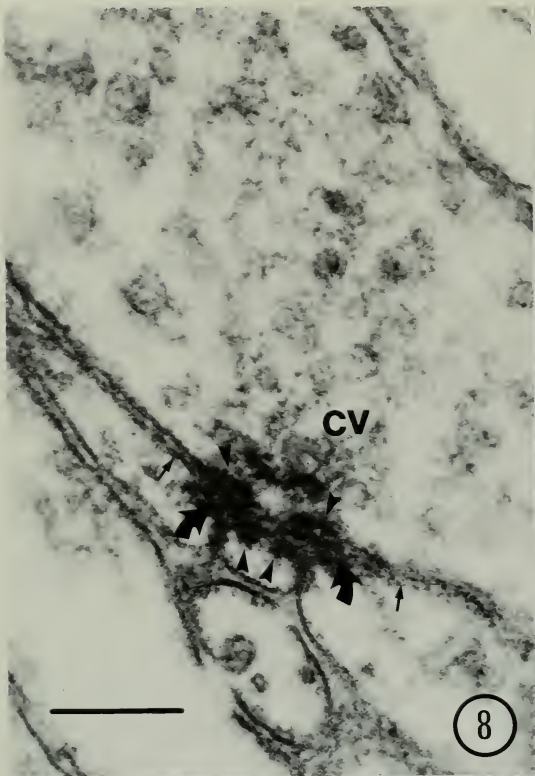
spheroidal vesicles (~30–50 nm) in the presynaptic terminal and presynaptic densities opposite the enzyme stain. The reaction products at these endings were found in the synaptic cleft only between the synaptic contact zone. The association of the AChE enzyme reaction product with synaptic endings that contain clear spheroidal synaptic vesicles has also been clearly demonstrated in vertebrates (Spencer and Baker, 1986) and in higher invertebrates (Smith and Treherne, 1965; Newman et al., 1968). In well-studied vertebrate models, the presence of clear spheroidal synaptic vesicles at axon terminals plus the colocalization of AChE and choline acetyltransferase is indicative of cholinergic function. Nevertheless, in *Fasciola*, AChE activity was not always found at synaptic sites containing the clear spheroidal synaptic vesicles and the enzyme activity was frequently localized in areas of no synaptic activity.

The identification of a neurotransmitter in any organism has several requirements including the demonstration of a physiological effect of the putative neurotransmitter; the presence of the compound, its precursors, and all the enzymes for synthesis and degradation of the neurotransmitter; and the localization of the neurotransmitter compound or associated enzymes in the appropriate sites (Gainer and Brownstein, 1981; Shepherd, 1983). *Fasciola hepatica* has the biochemical machinery to synthesize and degrade ACh, and exogenously applied ACh has a receptor-mediated physiological effect on the parasite (Sukhdeo et al., 1986). Acetylcholinesterase has been quantitatively demonstrated (Sukhdeo et al., 1986) and has been previously localized in nervous tissues at the light microscope level (Krvavica et al., 1967; Panitz and Knapp, 1970). This study on the ultrastructural localization of AChE provides strong evidence that ACh acts as a neurotransmitter in this flatworm parasite. Electrophysiological proof of neurotransmitter function is not yet technically feasible in these organisms but would provide final evidence.

The Platyhelminthes, the phylum to which *F.*

FIGURES 6, 7. 6. Cross-sectional profile of nerve processes in the neuropile. The enzyme reaction product appears as discrete patches (arrows) in the extracellular spaces. The distribution of the reaction product was not correlated with a specific morphological type of vesicle in the nerve processes. No reaction product is observed intracellularly associated with vesicles. mt, microtubules; cv, clear vesicles; dc, dense-core vesicles; m, mitochondria. Scale bar = 0.5  $\mu$ m. 7. Section through the neuropile, comparable to Figure 6, preincubated with BW284C51. In areas where reaction product would normally occur extracellularly, none were found (arrows). cv, clear vesicles; dc, dense vesicles; s, synapse with no reaction product; large arrowhead, microtubules. Scale bar = 1  $\mu$ m.





*hepatica* belongs, is considered to possess the first "true" brains. However, it is becoming increasingly evident from behavioural, physiological, and anatomical studies that much of the basic neural machinery seen in complex central nervous systems is already present in members of this phylum (Morita and Best, 1966; Koopowitz and Chien, 1975; Koopowitz and Keenan, 1982; Bullock, 1984; Sukhdeo and Mettrick, 1987). Several putative neurotransmitters (ACh, 5-HT, dopamine, glutamate) have been reported from these organisms (Welsh and Moorhead, 1960; Hillman et al., 1976; Bennett and Gianutsos, 1977; Keenan and Koopowitz, 1982; Hauser and Koopowitz, 1987), but much of the evidence for these conclusions was based on histofluorescence or histochemical techniques and the neurotransmitter functions of these compounds have not been conclusively demonstrated. The evidence in this study shows that the patterns and location of AChE in the nervous system of *F. hepatica* are very similar to those seen in higher invertebrates and vertebrates. These results suggest that the basic mechanisms of neurotransmission in the higher vertebrates and invertebrates were already present in this primitive phylum.

#### ACKNOWLEDGMENTS

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FIGURES 8–11. 8. Axonal synaptic contact zone characterized by pre- and postsynaptic densities (arrowheads) and an accumulation of clear vesicles (cv) near to the synaptic site. The AChE reaction product is found between the zone of the synaptic contact (curved arrows) and is absent outside this zone (small arrows). Scale bar = 0.2  $\mu$ m. 9. Triad- or wedge-type synapse showing AChE reaction product in the synaptic cleft between the contact zone (curved arrows) but not elsewhere (small arrows). This section was not poststained to enhance the black reaction product but resulted in loss of structural details because of the lack of contrast. cv, clear vesicles; m, mitochondria. Scale bar = 1  $\mu$ m. 10. Two triad- or wedge-type synapses from 1 nerve process (P1) that demonstrate the reaction product stain in the extracellular space at the synaptic contact zones (curved arrows). Both synapses are characterized by the clustering of clear vesicles (cv). There is another nerve process (P2) with 2 synapses (curved arrows) that also contain the reaction product in the extracellular space and these also have clear vesicles. m, mitochondria. Scale bar = 0.25  $\mu$ m. 11. A morphologically similar type of synaptic ending to that shown in Figure 8. No AChE reaction product is seen in the synaptic contact zone (curved arrows). However, reaction product stain is located in the extracellular space associated with this process even though it is not at the synaptic site (arrowhead). cv, clear vesicles; dv, dense vesicles. Scale bar = 0.2  $\mu$ m.



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# RESEARCH NOTES

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## *Sarcocystis arieticanis* and Other *Sarcocystis* Species in Sheep in the United States

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**ABSTRACT:** Histological sections of tongues, esophagi, and diaphragms from 512 adult ewes from the north-west United States and Texas were examined for *Sarcocystis* spp. Sarcocysts were found in sections of 82.1% of 504 tongues, 44.4% of 478 esophagi, and 51.7% of 89 diaphragms. *Sarcocystis tenella* was the predominant species and was found in 430 (84.0%) sheep; *S. arieticanis* was found in 18 (3.5%) sheep. The mean number of *S. tenella* sarcocysts in tissue sections was approximately 10 times higher than that of *S. arieticanis*. The identification of *S. arieticanis* was confirmed by ultrastructural studies and by transmission to dogs. Macroscopic sarcocysts of *S. gigantea* were also found but were not quantitated in all sheep; sarcocysts of *S. medusiformis* were not observed.

Four species of *Sarcocystis* are reported from domestic sheep: *Sarcocystis tenella*, *S. arieticanis*, *S. gigantea*, and *S. medusiformis* (Levine, 1986). The first 2 species are transmissible via canids. The third and fourth species are transmissible via felids. *Sarcocystis tenella* and *S. arieticanis* form microscopic sarcocysts and are pathogenic for sheep, whereas *S. gigantea* and *S. medusiformis* form macroscopic sarcocysts and are not pathogenic (Dubey et al., 1988). However, carcasses infected with macroscopic sarcocysts are condemned in part or in whole, resulting in losses to producers. *Sarcocystis tenella* and *S. gigantea* have been reported from many countries, including the U.S., and probably have worldwide distributions. *Sarcocystis arieticanis* has been reported only from the Federal Republic of Germany, German Democratic Republic, New Zealand, and Australia (Bergmann and Kinder, 1975; Boch et al., 1979; Erber, 1982; Heydorn, 1985; O'Donoghue and Ford, 1986; Pomroy and Charleston, 1987). *Sarcocystis medusiformis* has been reported only from New Zealand and Australia (Collins et al., 1979; Obendorf and Munday, 1987). We report herein the prevalence of the *Sarcocystis* species occur-

ring in adult ewes raised in the western United States and Texas.

Between March and May of 1983, 5 shipments (groups 1-5) of whole tongues and esophagi from 364 2-8-yr-old ewes were transported on ice from the Kachina Packing Company, Gallup, New Mexico, to the Animal Parasitology Institute (API), Beltsville, Maryland. These ewes had reportedly been raised in Colorado and Idaho. Ovine tongues and esophagi were inspected for grossly visible sarcocysts as reported earlier (Dubey et al., 1986). Between March 1984 and June 1985, 3 additional shipments (groups 6-8) of tongues and esophagi from 148 ewes > 5 yr old were received from Swift Independent packing plant in San Angelo, Texas. Diaphragms were also obtained from ewes in groups 7 and 8; all 148 ewes had been raised in Texas. Their tissues were examined visually for macroscopic sarcocysts but none were found.

Because we did not find macroscopic sarcocysts in the first 148 ewes from Texas, we were interested in determining if macroscopic sarcocysts existed in Texas sheep. Therefore, efforts were made to obtain macroscopic sarcocysts from naturally infected Texas sheep. On 21 June 1985, 10 esophagi and 6 diaphragms containing grossly visible sarcocysts were received from San Angelo, Texas. The esophageal sarcocysts were grossly and ultrastructurally identical with those of *S. gigantea* and were not investigated further (Collins et al., 1979). The 10 macroscopic sarcocysts in the diaphragms were much more slender (5-8 × 1 mm) than esophageal sarcocysts and appeared similar to those of *S. medusiformis*. Therefore, further identification of these sarcocysts from the diaphragms was attempted. Three of these macroscopic sarcocysts were fixed in 2% glutaraldehyde in Millonig's buffer and processed for transmission electron microscopy (TEM)



(Speer and Dubey, 1982), and 1 was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6  $\mu\text{m}$  thickness, and stained with hematoxylin and eosin (H&E) for light microscopy. The remaining 6 macroscopic sarcocysts were dissected free from the host tissue, rinsed several times in saline (0.9% NaCl) solution, and fed to a *Sarcocystis*-free cat. The cat came from the cat colony at the API and had never been fed uncooked meat. The cat shed a few sporocysts intermittently from 15 to 20 days later and was euthanatized 21 days after ingesting the 6 macroscopic sarcocysts. Sporocysts were collected from intestinal scrapings as described by Dubey (1980). A 15-day-old laboratory-raised lamb was fed approximately 10,000 sporocysts and euthanatized 692 days after inoculation (DAI); clinical signs were not seen in this inoculated lamb.

Portions of tongues and esophagi from sheep in group 1 were pooled, ground, and fed to a *Sarcocystis*-free dog. The dog shed sporocysts 6 days later and was euthanatized 19 days after ingesting the mutton. Sporocysts were collected from intestinal scrapings of the dog and 10,000 sporocysts were fed to a 4-mo-old sheep from the API flock. This sheep developed clinical signs of acute sarcocystosis but survived and was euthanatized and examined at necropsy 103 DAI.

Tissues from naturally infected sheep were processed for routine histologic examination. Tissues from groups 1 to 5 were fixed 1–3 days after slaughter, whereas those from groups 6 to 8 were fixed within 2 hr of slaughter. Tissues were kept cool between the times of slaughter and fixation. Experimentally infected sheep were necropsied and portions of all internal organs were fixed and processed for histologic examination (Dubey et al., 1982). Selected tissue sections were stained with periodic acid Schiff–hematoxylin (PAS) and silver stains. All sarcocysts present in a tissue section of tongue ( $3 \times 2$  cm), esophagus ( $3 \times 0.5$  cm), and diaphragm ( $3 \times 0.5$  cm) from each of the naturally infected sheep were examined under oil immersion to determine the species of *Sarcocystis* present. Sarcocysts with striated walls 1–3  $\mu\text{m}$  thick were considered to be those of *S. tenella*, those with  $<1$ - $\mu\text{m}$ -thick walls with hairlike projections were considered to be those of *S. arieticanis*, and those with no structural features present that would allow identification were considered to be unidentifiable.

To compare the efficiency of histologic examination versus digestion procedures, approx-

imately 50 g of each tongue from sheep in groups 7 and 8 were homogenized in a blender and digested in a  $10\times$  volume of acid-pepsin solution as described by Jacobs et al. (1960). After a 90-min incubation at 37 C, the digest was filtered through cheesecloth and centrifuged at 400 g for 10 min. After discarding the supernatant fluid, the pellet was mixed in 10 ml of saline solution and a drop (approximately 0.03 ml) was examined microscopically for *Sarcocystis* bradyzoites.

Sarcocysts (Figs. 1–5) were found in histologic sections from 430 of 512 sheep (Table 1). Both the intensity of infection and the prevalence were higher in the tongue than in the esophagus or diaphragm. *Sarcocystis tenella* (Fig. 3) was the predominant species and was found in 430 (84.0%) of the sheep. *Sarcocystis arieticanis* (Figs. 1, 2) was found in 18 (3.5%) of the sheep; usually only a few sarcocysts were present. It was not possible to identify every sarcocyst in many tissue sections because of the angle of the sarcocyst sectioned or other alterations to sarcocyst structure that occurred during fixation and tissue processing. Two thick-walled sarcocysts (Fig. 4), unlike any previously described from sheep, were seen in the tongues of 2 sheep from groups 3 and 4. These sarcocysts were immature and measured  $93.8 \times 38.3$   $\mu\text{m}$  and  $75.0 \times 32.5$   $\mu\text{m}$ . Their walls were 5.0–7.5  $\mu\text{m}$  thick and had dense hairlike projections.

The macroscopic sarcocysts from the diaphragm of the naturally infected sheep from Texas were identified as those of *S. gigantea* (Fig. 5) based on structure and biology. The diaphragmatic sarcocysts had a connective tissue capsule around them (Fig. 5) and the villar protrusions on the cyst wall were cauliflower-like, identical to those described by Collins et al. (1979). In the sheep examined 692 DAI with sporocysts obtained from the cat fed macroscopic sarcocysts, sarcocysts 1–3 mm in length were found in the esophagus and laryngeal area (7 sarcocysts) and the diaphragm (1 sarcocyst). The structures of these macroscopic sarcocysts were identical to each other and to that reported for *S. gigantea*.

Both *S. tenella* and *S. arieticanis* sarcocysts were found in skeletal muscles of the sheep inoculated with sporocysts from the dog. Isolated unstained sarcocysts of *S. arieticanis* had hairlike projections, whereas those of *S. tenella* had villar projections. Four structurally distinct sarcocysts were found in histologic sections. *Sarcocystis arieticanis* was thin-walled with or without hairlike projections (Figs. 1, 2). The reason for this discrepancy became clear after exami-

TABLE 1. Prevalence, mean number, and identity of sarcocysts from tissue sections of 8 groups of naturally infected sheep.

Group	Number of sheep	Total infected (%)	<i>Sarcocystis tenella</i>			<i>Sarcocystis arieticanis</i>			Unidentified		
			T*	E	D	T	E	D	T	E	D
1	56	55 (98.2)	25.1 (54)†	2.0 (29)	NE	0	0	NE	5.6 (53)	1.0 (7)	NE
2	50	50 (100)	19.0 (48)	3.1 (29)	NE	3.0 (2)	1.0 (1)	NE	2.9 (37)	1.2 (13)	NE
3	100	99 (99.0)	25.9 (98)	3.6 (48)	NE	1.0 (1)	0	NE	6.0 (88)	1.3 (19)	NE
4	57	57 (100)	42.6 (57)	3.5 (38)	NE	0	0	NE	10.6 (57)	1.3 (13)	NE
5	101	69 (68.3)	17.9 (67)	3.0 (22)	NE	4 (1)	0	NE	4.5 (49)	1.2 (10)	NE
6	50	34 (68.0)	4.8 (33)	6.4 (7)	NE	0	0	NE	2.0 (7)	1.3 (3)	NE
7	48	29‡ (60.4)	20.0 (18)	9.7 (12)	13.8 (16)	7.4 (11)	14.3 (3)	22.8 (5)	6.2 (13)	4.1 (7)	7.2 (12)
8	50	37§ (74.0)	12.7 (31)	3.4 (10)	8.0 (27)	2.0 (3)	1.0 (1)	0	4.4 (18)	1.3 (3)	3.2 (12)

\* T = tongue, E = esophagus, D = diaphragm.

† Number of positive sheep used to calculate the mean. NE = not examined.

‡ Digest of tongues from 46 sheep in this group revealed 91.3% infected.

§ Digest of tongues from 47 sheep in this group revealed 97.8% infected.

nations of TEM photomicrographs of *S. arieticanis*. Figure 6 shows a sarcocyst of *S. arieticanis* with an uneven cyst wall that has barely visible protrusions at some places and prominent protrusions at other places (Figs. 6, 7).

Ultrastructurally, the primary cyst wall (PCW) of *S. arieticanis* consisted of a parasitophorous vacuolar membrane (PVM) with an inner electron-dense layer immediately beneath it. The PCW formed long tubular projections (6.7–10  $\mu$ m long), which consisted of a dome-shaped base (0.4–0.6  $\mu$ m in diameter), a relatively thick midregion (0.4–3.0  $\mu$ m), and a thin distal region (0.03  $\times$  3–6.8  $\mu$ m) (Fig. 6). Immediately above the dome-shaped base, the projections turned 90° to the surface of the sarcocyst so that the midregion and distal region were oriented nearly parallel to the sarcocyst surface. At various locations around the sarcocyst surface, the projections were arranged in a conical structure that extended as much as 7–9  $\mu$ m into the cytoplasm of the host cell (Fig. 7). The PVM was highly convoluted in the dome-shaped base, forming indentations that were 0.04  $\mu$ m in diameter. A granular layer 0.24  $\mu$ m thick was situated between the PCW and the zoites of the sarcocyst. No septa were observed within the sarcocyst. The sarcocysts contained numerous bradyzoites and few merozoites near the margin of the cyst. The bradyzoites and merozoites contained all the organelles characteristically found in those of other *Sarcocystis* species.

*Sarcocystis* bradyzoites were found in 91.3% (group 7) and 97.8% (group 8) of the 2 groups of tongues digested. The corresponding identifications by microscopic examination of H&E-stained tissue sections of tongues were 46.8% and 71.1%, respectively.

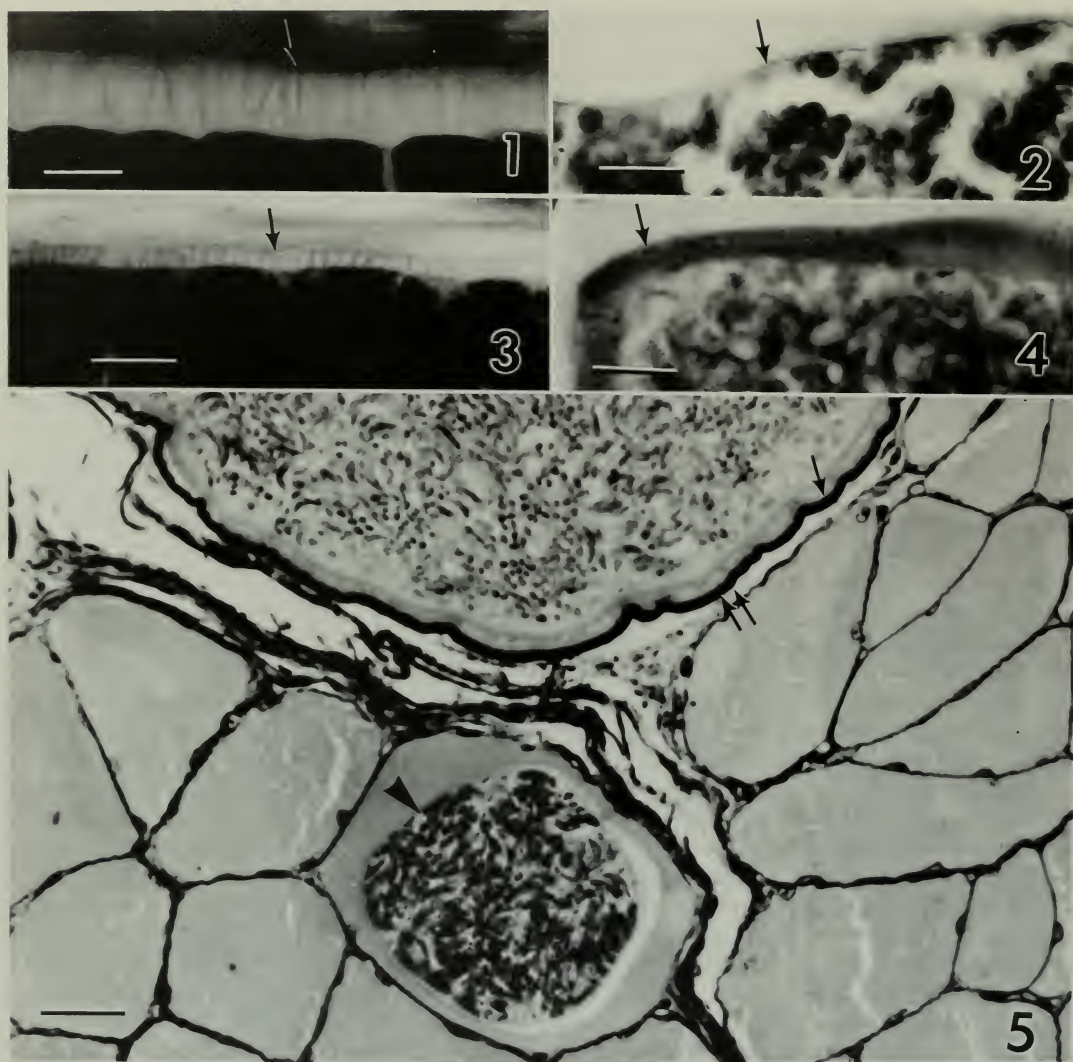
*Sarcocystis tenella* is the most pathogenic of all ovine *Sarcocystis* species and was the most

prevalent species in this survey. *Sarcocystis* species were found in digests of 91.3% and 97.8% of the sheep examined with this technique. This high prevalence in old (> 5 yr) ewes is surprising and suggests that either sheep are reinfected continuously or that sarcocysts persist for several years in sheep. Reinfection is more likely to be the case because the environment is highly contaminated with sporocysts, the lambs become infected before weaning, and there is only partial immunity to reinfection (Leek and Fayer, 1980; Ford, 1985). In experimentally infected sheep, sarcocysts began to decrease 3 mo postinoculation and new sarcocysts were formed from challenge infections (Ford, 1985).

*Sarcocystis arieticanis* is reported for the first time in sheep from the U.S. It was much less common than *S. tenella*, and in the Federal Republic of Germany Boch et al. (1979) found *S. arieticanis* (syn., *Sarcocystis* sp.) in 84.8% of 500 sheep from Bavaria. These differences may be related to the age of sheep surveyed and the techniques employed. Our observations are based on histologic examination of H&E-stained tissue sections, whereas others made their observations on live preparations. In this respect, we could not identify all the sarcocysts in many tissue sections; some of these unidentified sarcocysts might have been those of *S. arieticanis*.

We found the cyst wall of *S. arieticanis* to be similar in appearance to electron micrographs presented for this same species of *Sarcocystis* by Heydorn and Mehlhorn (1987) but we found no septa and therefore no compartments in the 3 cysts examined ultrastructurally 103 DAI. Also, Heydorn and Mehlhorn (1987) reported that the hairlike protrusions occurred over the surface of the sarcocyst and were longest (approximately 11  $\mu$ m) at its tips. We found that the protrusions





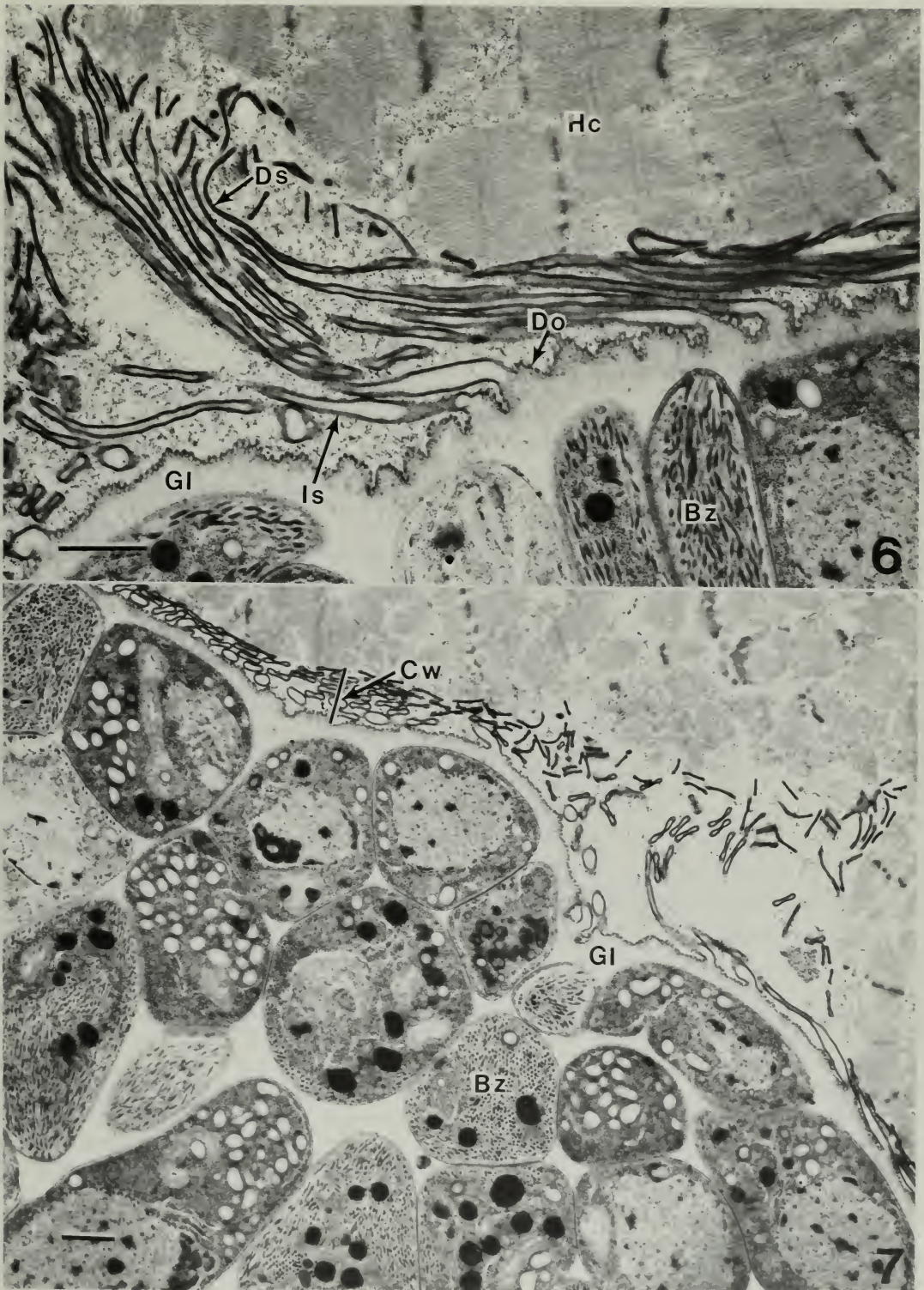
FIGURES 1-5. Sarcocysts in skeletal muscles of sheep. Single arrows point to outer limit of the sarcocyst wall. 1. Sarcocyst of *S. arieticanis* with hairlike projections. H&E stain, 103 DAI. Bar = 10  $\mu$ m. 2. Sarcocysts of *S. arieticanis* with smooth cyst wall. H&E stain, 103 DAI. Bar = 10  $\mu$ m. 3. Sarcocyst of *S. tenella* with stubby villar projections. H&E stain, 103 DAI. Bar = 10  $\mu$ m. 4. Sarcocyst of an unidentified *Sarcocystis* sp. with a thick wall with dense hairlike projections. H&E stain, natural infection. Bar = 10  $\mu$ m. 5. Sarcocyst of *S. gigantea* showing the connective tissue capsule (double arrows) that surrounds its wall. Note that the sarcocyst of *S. arieticanis* (arrowhead) from a natural infection lacks a connective tissue capsule. Silver stain, 692 DAI. Bar = 10  $\mu$ m.

were similar in length over the entire sarcocyst surface and that in certain areas the protrusions were arranged collectively to form conical tufts. In areas removed from these conical tufts, the cyst wall appeared thinner (1-3  $\mu$ m thick) because the protrusions were bent 90° so that they were oriented parallel to the sarcocyst surface.

The 4 named species of *Sarcocystis* in sheep can be distinguished by the structure of their sarcocyst walls. *Sarcocystis arieticanis* is thin-walled with hairlike protrusions and has no sep-

ta. *Sarcocystis tenella* is thick-walled with stubby villar protrusions. *Sarcocystis gigantea* is thin-walled, surrounded by a connective tissue capsule and has highly branched cauliflower-like villar protrusions. *Sarcocystis medusiformis*, which was not found in this survey, is thin-walled with highly branched snakelike villar protrusions. A fifth structural type of sarcocyst, with an unusually thick wall, was found in 2 sheep. Because the sarcocysts were not mature and were rarely found, they could not be speciated.





FIGURES 6, 7. Transmission electron micrographs of *S. arieticanis* sarcocysts in the skeletal muscle of an experimentally infected sheep, 103 DAI. 6. Sarcocyst wall showing hairlike protrusions; Bz, bradyzoite; Do, dome-shaped base of protrusion; Gl, granular layer; Hc, host cell cytoplasm; Is, intermediate segment of protrusion. Bar = 1  $\mu$ m. 7. Portion of a sarcocyst showing lack of septa; Bz, bradyzoite; Cw, cyst wall; Gl, granular layer. Bar = 1  $\mu$ m.



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## Nematode Infestation of Fillets from Atlantic Cod, *Gadus morhua*, Off Eastern Canada

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**ABSTRACT:** The prevalence and intensity of larval nematodes in fillets of Atlantic cod, *Gadus morhua*, were examined and compared with similar data from a previous survey conducted about 30 yr ago. *Anisakis simplex* occurred more often in the nape of the fillet, whereas *Pseudoterranova decipiens* was the predominant species in napeless fillets. The results suggest an increase in both the prevalence and intensity of *P. decipiens* in fillets of cod, especially those originating from the Gulf of St. Lawrence and coastal Nova Scotia.

Fillets of Atlantic cod, *Gadus morhua* L., and at least 6 additional species of fish, are infested

with nematodes, *Pseudoterranova* (*Phocanema*) *decipiens* and *Anisakis simplex* (see Templeman et al., 1957). During the past 7 yr, fish processors in Newfoundland and Nova Scotia have reported that the number of worms in fillets have increased considerably and consequently constitute not only a potential public health hazard but also a financial loss in marketing value. Ingestion of these worms, as a result of consuming improperly cooked or raw fish, may produce a disease condition called anisakiasis. Margolis (1977) reported several human cases following inges-

TABLE I. Comparisons of prevalence (%) and intensity (no./kg) of larval nematodes in fully-naped, half-naped, and napeless fillets of Atlantic cod, *G. morhua*, originating from different areas adjacent to Newfoundland (Nfld.) in 1947–1953 (Templeman et al., 1957) and in 1984–1985 (present study).

	1947–1953*			1984–1985			Increase in abundance
	n	% Prevalence	Intensity/kg	n	% Prevalence	Intensity/kg	
Gulf of St. Lawrence (NAFO subarea 4R)	599	39.7	1.18	215†	51.0	3.20 ± 0.16	2.7
				142‡	49.2	2.94 ± 0.29	2.5
				277	25.0	1.97 ± 0.43	1.7
West coast, Nfld. (NAFO subarea 3Pn)	935	30.4	1.40	45†	40.0	3.90 ± 0.24	2.9
				82‡	30.5	2.85 ± 0.15	2.0
Northeast coast (NAFO subarea 3K)	699	3.0	0.05	109†	18.4	0.85 ± 0.12	18.5
				59‡	8.5	0.22 ± 0.04	4.8
Scotian Shelf (NAFO subarea 4V)	533	17.0	0.64	116	40.0	9.20 ± 0.94	15.3
St. John's/Portugal Cove, Nfld.	1,611	3.7	0.07	275	9.0	0.15 ± 0.03	2.0
Fortune Bay, Nfld.	257	14.0	0.31	125	21.0	0.90 ± 0.04	2.9

\* Templeman et al. (1957); fillets examined appeared to be napeless.

† Fully-naped.

‡ Half-naped.

tion. The present study was undertaken to ascertain the prevalence of nematodes in cod taken in some areas in and adjacent to Newfoundland and to compare infestation levels with those reported by Templeman et al. (1957).

Cod were sampled inshore on the south (Fortune), west (Port aux Basques and Isle aux Morts), and east (Portugal Cove) coasts of Newfoundland; offshore sampling was conducted in the Gulf of St. Lawrence (North Atlantic Fisheries Organization [NAFO] subarea 4R) and on the continental shelf off Nova Scotia (Scotia Shelf, NAFO subarea 3K) and the northeast coast of Newfoundland. Only half-naped fillets were examined on the south and east coasts, whereas full-naped, half-naped, and napeless fillets were examined on the west coast. The nape is the dorsum of the "neck" area immediately posterior to the head (Fahey, 1983). Sampling of each fillet involved candling as well as slicing into smaller pieces (destructive sampling) during examination. All worms collected were preserved in 70% alcohol. Some were mounted in lactophenol and identified microscopically according to Templeman et al. (1957).

The highest prevalence of infection occurred in fully-naped fillets originating from the Gulf of St. Lawrence (51%) and the west coast of Newfoundland (40%) rather than from the northeast coast (18%). Similarly, a high percentage (49%) of the half-naped fillets from cod taken near the west coast of Newfoundland harbored worms in contrast to the Scotian Shelf (40%), Fortune Bay (21%), or the northeast coast, both inshore (9%) and offshore (9%). In all areas from which fillets

were examined, there was an increase in the number of worms per fillet compared with the numbers reported by Templeman et al. (1957). The mean intensity of nematodes increased substantially in cod taken from NAFO subarea 3K where it was 18.5 times the levels recorded between 1947 and 1953 (Templeman et al., 1957, vide Table I). Ninety-seven percent of the worms examined from napeless fillets were *P. decipiens*, whereas a higher prevalence (33%) of *A. simplex* occurred in fully-naped fillets. *Anisakis simplex*, predominant in the napes and belly flaps, were encysted in the flesh.

Results from the present study suggest that there has been a substantial increase of larval nematodes, especially *P. decipiens*, in the fillets of cod, which may be associated with increases in seal populations in the Gulf of St. Lawrence and Magdalen Islands off Nova Scotia. Grey (*Halichoerus grypus*) and harbour seals (*Phoca vitulina*) are the main definitive hosts of *P. decipiens* in these areas (McClelland, 1980). To avoid contamination of fillets sold commercially, it is recommended that the napes, which harbor a high percentage of nematodes, be excluded from cod taken off the Nova Scotian shelf and the Gulf of St. Lawrence. Additionally, when worm intensity exceeds 5 per fillet during initial examination by candling, the fillets (especially those from large fish [ $>60$  cm]) should be discarded, as closer examination invariably reveals additional worms. Processors should also be aware that thickness, intensity of bruising, degree of freshness, and improper skinning that results in the retention of a white membrane (connective



tissue), all impair detection of nematodes in a fillet.

This is Marine Sciences Research Laboratory contribution number 728.

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## Possible Involvement of Calcium Ions in the Hatching of *Schistosoma mansoni* Eggs in Water

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**ABSTRACT:** The possibility of involvement of calcium ions in the hatching of *Schistosoma mansoni* eggs in water is described. The hatching of *S. mansoni* eggs under low osmotic pressure was partially inhibited by EGTA (5 mM), lanthanum chloride (1-5 mM), and ruthenium red (0.1-1 mM). The reagents used in these experiments were not toxic to the eggs however, because miracidia hatched normally when the reagents were removed.

*Schistosoma* eggs hatch readily in fresh water, but few eggs hatch in physiological saline solution. The hatching mechanism of these eggs requires clarification, although some information on this subject does exist (Kusel, 1970; Bair and Etges, 1973; Kassim and Gilbertson, 1976; Samuelson et al., 1984; Xu and Dresden, 1986). Matsuda et al. (1983) reported that praziquantel (PZQ) stimulated the hatching of *Schistosoma japonicum* eggs even when the eggs were kept under high osmotic pressure. Recently, Katsumata (1988) reported that PZQ-induced hatching was inhibited by  $\text{Ca}^{2+}$  channel blockers and EGTA, and that the calcium ionophore (A23187) stimulated the hatching of *Schistosoma mansoni* eggs under high osmotic pressure. In this study, we examined the effect of  $\text{Ca}^{2+}$  channel blockers and EGTA on the hatching of *S. mansoni* eggs placed under low osmotic pressure.

The Kenyan strain of *S. mansoni* was used in this study. Eggs were obtained from the livers of

hamsters by a digestion technique (Katsumata, 1988). The eggs were suspended in 1.1% NaCl solution (365 mOsm). Ten  $\mu\text{l}$  of egg suspension (containing about 150 eggs) were mixed with 1 ml of test solution (aqueous solution of ruthenium red [RR], lanthanum chloride [ $\text{La}^{3+}$ ], and EGTA) and with 1 ml of low osmolarity control solution (0.09% NaCl). The osmotic pressure and pH of aliquots of the mixed solutions were determined. The osmotic pressure was measured with a Fiske Osmometer OS (Fiske). Eggs were allowed to hatch for 2 hr at 25 C, and then fixed with formalin. In each solution, the ratio of hatched miracidia to the total number of mature eggs was determined. Eggs with a normal appearance and clearly outlined miracidia were arbitrarily identified as mature eggs. In our preparation, approximately 70% of the eggs were mature. The osmotic pressures of the test solutions were adjusted to slightly lower levels than those of controls because of technical difficulties. In addition, the osmotic pressures of low reagent concentration test solutions were adjusted to levels slightly higher than those of high reagent concentration solutions by adding 0.09% NaCl solution. The osmotic pressure and pH of test solutions used in these experiments are described in Table I. To examine the effect of pH on the hatching of eggs, eggs were allowed to hatch in dilute phosphate-buffered saline, pH 7.4, and di-

TABLE I. Effect of lanthanum chloride ( $\text{La}^{3+}$ ), ruthenium red (RR), and EGTA on hatching of *S. mansoni* eggs under low osmotic pressure.\*

Incubation medium			No. of miracidia/(no. of mature eggs unhatched + no. of miracidia) (% hatch)	Average % hatch†
Reagents	Osmolarity (mOsM)	pH		
Diluted glycine-HCl buffer	31	4.0	111/152 (73), 126/174 (72), 149/190 (78), 122/177 (69)	73a
0.09% NaCl	31	5.0	120/161 (75), 113/154 (73), 103/148 (70), 125/181 (69)	72a
Diluted PBS	31	7.4	154/202 (76), 71/105 (68), 120/178 (67), 141/201 (70)	70a
0.1 mM RR	29		71/120 (59), 54/117 (46), 61/154 (40), 47/124 (38)	46b
0.5 mM RR	26		55/160 (34), 42/163 (26), 29/123 (24), 71/195 (36)	30c
1 mM RR	19	4.9	17/100 (17), 24/127 (19), 42/157 (27), 34/134 (25)	22c
0.1 mM $\text{La}^{3+}$	30		74/132 (56), 109/149 (73), 55/110 (50), 121/153 (79)	65a
1 mM $\text{La}^{3+}$	29		64/140 (46), 57/124 (46), 48/123 (39), 59/105 (56)	47b
5 mM $\text{La}^{3+}$	25	4.6	47/170 (28), 38/155 (25), 33/143 (23), 32/137 (23)	25c
5 mM EGTA	27	4.0	72/162 (44), 72/164 (44), 79/173 (46), 72/145 (50)	46b

\* Four replicates were done for each test solution. Eggs were incubated in solutions for 2 hr at 25 C.

† Means with the same letter are not significantly different ( $P < 0.01$ , Duncan's multiple range test).

lute glycine-HCl buffer, pH 4.0 (31 mOsM). Four replicates were done for each test solution.

Varying the pH did not have any effect on the hatching of eggs. In the 31 mOsM control solution (0.09% NaCl) approximately 70% of the mature eggs hatched in 2 hr. In solutions containing RR,  $\text{La}^{3+}$ , or EGTA, the hatching rate was reduced although the osmotic pressure of the test solution was lower than that of controls (Table I). The possible toxic effects of the reagents on the eggs were examined. Eggs were preincubated in 1 mM RR, 5 mM  $\text{La}^{3+}$ , and 5 mM EGTA solution for 2 hr (21–29 mOsM), washed repeatedly with 1.1% NaCl solution, and then allowed to hatch in 0.09% NaCl solution (31 mOsM) for 2 hr. Hatching rates of the 3 groups of eggs in reagent-free solution were 73, 74, and 79%, respectively. This reversible hatchability indicates that even the high concentration of reagents used in these experiments were not toxic to eggs.

Although the present report neither supports nor rejects the 3 previously proposed mechanisms of hatching in water, i.e., proteolytic enzyme, osmotic pressure, and mechanical activity of the miracidium, we conclude that a calcium-

mediated process might play an essential role in the hatching of *S. mansoni* eggs in natural water.

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## A Comparative Study of the Development of *Eimeria nieschulzi* In Vitro Under Aerobic and Reducing Conditions

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**ABSTRACT:** Sporozoites of the rat coccidian, *Eimeria nieschulzi* Dieben, 1924 (Apicomplexa: Eimeriidae), were inoculated onto monolayers of normal rat kidney (NRK) fibroblasts and cultured either under aerobic (5% CO<sub>2</sub>/95% air) or reducing (desiccator jars modified into candle jars) conditions in RPMI-1640 supplemented with 5% fetal bovine serum, sodium bicarbonate, and antibiotics. Under aerobic conditions, first-generation meronts were observed at 2 days post-inoculation (DPI) and, except for individual third-generation meronts that were seen at 5 and 6 DPI, no further development was noted. Under reducing conditions, however, first-generation meronts observed at 2-5 DPI underwent additional development to form second-generation meronts (3-5 DPI), third-generation meronts (3-7 DPI), and a small number of fourth-generation meronts (5-8 DPI). Both second- and third-generation meronts were abnormal, exhibiting gigantism although the merozoites produced appeared normal. The gradual degeneration of cell monolayers under reducing conditions prevented further observations beyond 8 DPI. These results suggest that atmospheric conditions play an important role in the development of *E. nieschulzi* and maintenance of reducing conditions may be one key to achieving enhanced development of some species of coccidia *in vitro*.

Although numerous investigators have achieved partial development of coccidia *in vitro*, only 3 species have been reported to develop from sporozoites to oocysts in cell culture. Doran (1982) and Speer (1983) have reviewed most of the literature on *in vitro* cultivation of *Eimeria tenella*, which is well known for its ability to develop successfully under artificial conditions. Current and Haynes (1984), Naciri et al. (1986), and Wagner and Prabhu Das (1986) have achieved complete development of *Cryptosporidium parvum* and Upton et al. (1984) were able to obtain unsporulated oocysts of *Caryospora simplex* when sporozoites were used as inocula. Thus, it is obvious that when compared to the vast numbers of described species of coccidia, considerable work still needs to be done on identifying factors necessary to achieve enhanced parasite development *in vitro*.

Trager and Jensen (1976) and Jensen and Trager (1977) first described the successful cultivation of *Plasmodium falciparum* *in vitro* using

candle jars. It was also mentioned, but not described in detail, that some development of *Eimeria* spp. was achieved using this method (Trager and Jensen, 1976; Jensen, 1983). Therefore, it was of interest to us to compare development of an *Eimeria* sp. under reducing and nonreducing conditions in order to more closely examine the feasibility of using such a system for studies in our own laboratory.

Collection and concentration of oocysts of *E. nieschulzi* have been described elsewhere (Bristol et al., 1983). After exposing oocysts to 5% (v/v) Clorox® on ice for 5 min to kill microbial contaminants, oocysts were washed 4× in phosphate-buffered saline (PBS) and the walls removed with a motor-driven, teflon-coated tissue grinder. Following incubation for 50 min in an excystation solution consisting of 0.25% (w/v) trypsin-0.75% (w/v) sodium taurocholate in PBS, sporozoites were purified by passage through a nylon wool column (Tilahun and Stockdale, 1982).

Normal rat kidney (NRK-52E; ATCC CRL 1571) fibroblasts, a continuous cell line, were grown to near confluency on coverslips in 6-well tissue culture plates using RPMI-1640 media supplemented with 5% fetal bovine serum (FBS), Na-bicarbonate to pH 7.4, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml fungizone®. Each well was then inoculated with 1 × 10<sup>5</sup> sporozoites and the plates incubated at 37°C with 100% humidity in either a 5% CO<sub>2</sub>/95% air atmosphere or a reducing atmosphere in desiccator jars utilized as candle jars. At 24-hr intervals, some coverslips were examined for the presence of parasite development using Nomarski interference-contrast microscopy, whereas others were fixed in methanol and stained with Giemsa. All experiments were replicated a minimum of 3 times for each interval and measurements were made on 10 living parasites for each stage using a calibrated ocular micrometer.

The results of the study and measurement data for each of the stages observed are presented in Tables I and II, respectively. Sporozoites pene-

trated cells and, after 24 hr, both types of cultures contained >50 sporozoites/coverslip, although those incubated under aerobic conditions had notably fewer sporozoites within cells than those in reducing conditions. Some sporozoites (Fig. 1) transformed into spherical uninucleate-multinucleate meronts (trophozoites) (Figs. 2–5) within 24 hr and, by 2 days postinoculation (DPI), first-generation meronts were seen in both types of cultures (Fig. 6). Those cultures incubated under aerobic conditions had few (<10) meronts/coverslip, which disappeared rapidly so that no meronts were normally observed beyond 2 DPI, although individual third-generation meronts were noted at 5 and 6 DPI. These first-generation meronts were generally smaller and contained fewer merozoites than those reported *in vivo* (Roudabush, 1937; Marquardt, 1966), but could be easily distinguished by the presence of 2 small refractile bodies on either side of the nucleus within each of the merozoites.

Parasite development under reducing conditions proceeded further than in cultures incubated under aerobic conditions. Intracellular sporozoites (Fig. 1), trophozoites (Figs. 2–4), first- (Fig. 6), second- (Fig. 7), and third-generation (Fig. 8) meronts were seen in semi-anaerobic cultures at 3–5 DPI. Following host cell penetration, sporozoites rounded up and the refractile bodies fused to form a nearly spherical mass (Figs. 2–4). As merogony proceeded, the refractile material fragmented and became incorporated into the developing merozoites so that each merozoite contained a small refractile body on either side of the nucleus (Figs. 5, 6). Although merozoites arose from a single residuum by ectomerogony (Fig. 6), mature meronts rarely contained residual material.

Although individual merozoites appeared normal, most second-generation meronts themselves were aberrant, exhibiting gigantism with >60 merozoites per meront as opposed to 8–12 reported *in vivo* (Roudabush, 1937; Marquardt, 1966) (Fig. 7). These merozoites were more elongate than first-generation merozoites and contained granular material on either side of the nucleus (Fig. 7). Third-generation meronts also exhibited a high degree of gigantism (Fig. 8), with some meronts containing up to 60 merozoites while others appeared normal with 8–20 merozoites. These merozoites could be distinguished from the second-generation meronts only because they were more elongate. Although some second- and third-generation meronts had multiple residua indicating that more than 1 mero-

zoite probably initiated merogony from the same cell, other meronts had only 1 residuum, suggesting that some giant meronts may also have been formed from single merozoites. Both second- and third-generation meronts arose by ectomerogony and most mature meronts contained no residua.

Cells grown under reducing conditions began to vacuolate around 5 DPI, but these could sometimes be cultured to day 7, rarely to day 8, before sloughing of the cells occurred to such a degree that observations became impossible. At 5–8 DPI, occasional fourth-generation meronts were seen (Fig. 9). However, these were always found to be in poor condition due to the reduced atmospheric conditions and, thus, internal details of these meronts and merozoites could not be accurately observed. They were considered to be fourth-generation meronts only because the merozoites were shorter and more arc-shaped than merozoites of other generations. No gamonts were noted in our study.

To our knowledge, previous *in vitro* studies on *E. nieschulzi* are limited to a single report by Speer et al. (1970). These authors succeeded in culturing first-generation meronts of the parasite in embryonic bovine intestine, kidney, and liver cells. Whether additional development was achieved is not known, because their study appeared to be concerned with stimulation of motility of first-generation merozoites only.

Although sporozoites penetrated cells under both atmospheric and reducing conditions, we estimated (but did not quantitate) that perhaps

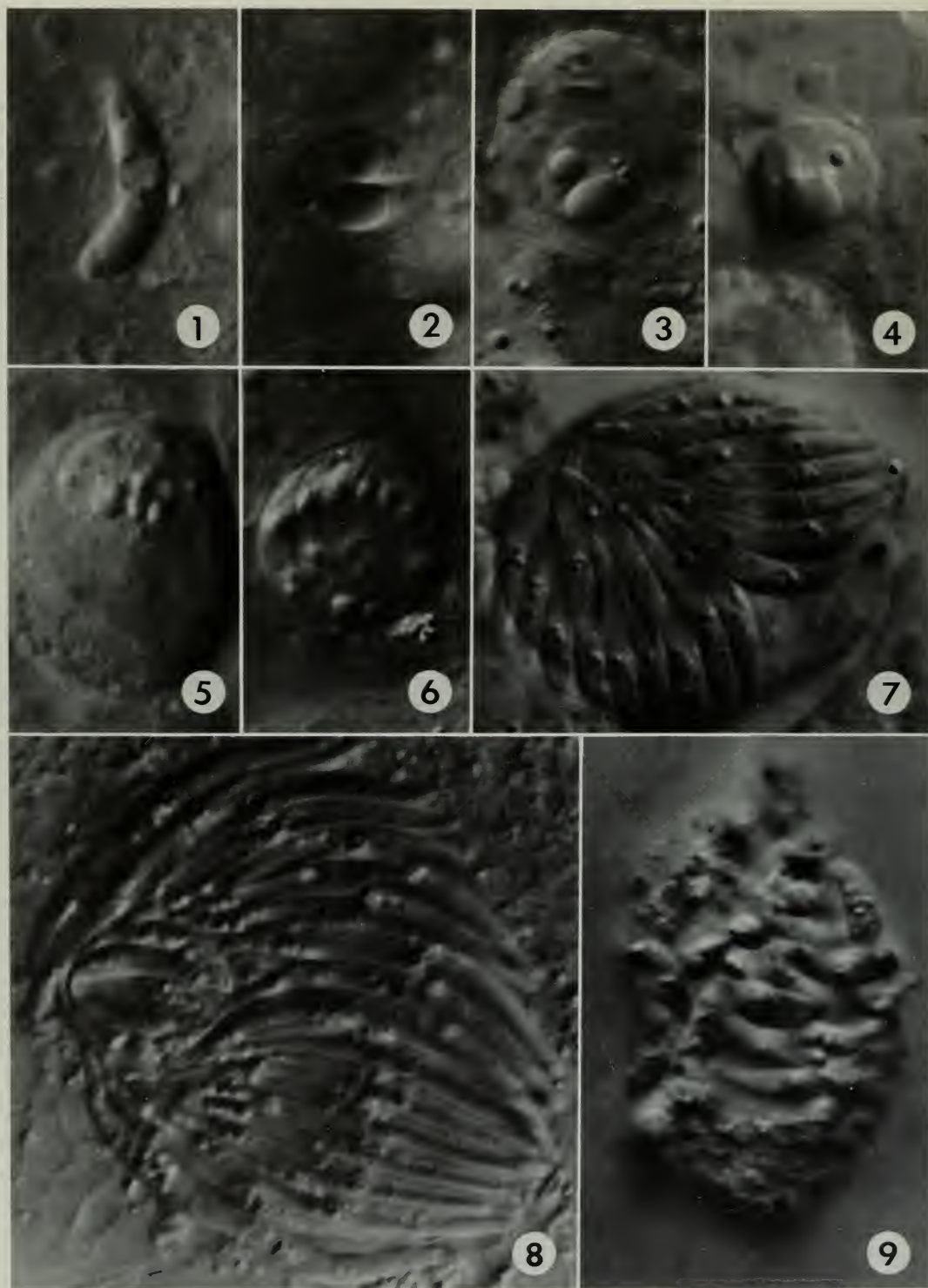
TABLE I. *Developmental stages of Eimeria nieschulzi in rat NRK fibroblasts.\**

Atmosphere	DPI	No. replicates	Sporozoites	Generation of meronts			
				I	II	III	IV
Aerobic	1	3	+++	—	—	—	—
	2	11	+++	+	—	—	—
	3	7	++	—	—	—	—
	4	6	+	—	—	—	—
	5	4	±	—	—	+	—
	6	5	—	—	—	+	—
	7, 8	4, 3	—	—	—	—	—
Reducing	1	7	+++	—	—	—	—
	2	11	+++	++	—	—	—
	3	11	+++	++	+	+	—
	4	7	++	++	+	++	—
	5	13	—	+	+	++	+
	6	7	—	—	—	++	+
	7	8	—	—	—	+	+
	8	6	—	—	—	—	±

\* No stages seen (—); present but degenerate (±); ≤10/coverslip (+); 11–50/coverslip (++); >50/coverslip (+++).

† A single meront present only among all coverslips.





FIGURES 1-9. Nomarski interference-contrast photomicrographs of developmental stages of *Eimeria nieschulzi* in normal rat kidney (NRK) fibroblasts under reducing conditions. All figures  $\times 2,200$ . 1. Sporozoite, 2 DPI. 2, 3. Sporozoites becoming spherical and transforming into first-generation meronts, 2 DPI. 4. Undifferentiated first-generation meront with large refractile body material, 2 DPI. 5. Undifferentiated first-generation meront with fragmenting refractile body material, 2 DPI. 6. Nearly mature first-generation meront with merozoites, 2 DPI. 7. Mature first-generation meront with merozoites, 2 DPI. 8, 9. Mature first-generation meronts with merozoites, 2 DPI.

TABLE II. Measurements (in  $\mu\text{m}$ ) of the living developmental stages of *Eimeria nieschulzi* in rat NRK fibroblasts.\*

Generation of meront	DPI	Atmosphere	No. merozoites per meront	Meronts	Merozoites†
First	2	Aerobic	16–20	15.2 × 13.2 (12.8–18.0 × 12.0–16.0)	13.6 × 1.8 (12.0–16.0 × 1.6–2.0)
First	2	Reducing	16–24	13.3 × 13.2 (12.0–16.0 × 11.2–16.0)	11.7 × 2.2 (11.0–12.0 × 2.0–2.4)
Second	3, 4	Reducing	>60	26.0 × 22.2 (20.0–40.0 × 14.4–31.2)	16.1 × 1.8 (15.2–16.8 × 1.8–2.0)
Third	5	Reducing	8–60	24.8 × 13.8 (20.0–36.0 × 10.4–18.4)	21.8 × 1.9 (20.0–24.8 × 1.8–2.2)
Fourth	6, 7	Reducing	30–60	22.0 × 20.6 (14.4–28.0 × 14.4–26.0)	9.3 × 2.1 (7.2–11.2 × 1.8–2.2)

\* n = 10 for each measurement.

† *In situ*.

a 50-fold increase in the number of intracellular parasites occurred under the reducing conditions. Subsequent development past first-generation meronts was inhibited in aerobic cultures, implying that  $\text{O}_2$  may have a deleterious effect on parasite development, although other factors such as pH may play important roles in affecting development of *E. nieschulzi* *in vitro*. Whether or not parasite development would have continued in candle jars if cell monolayers had not degenerated is not known, and media changes necessary for continued observations could not be performed because preliminary studies revealed that opening candle jars even briefly to change media resulted in loss of parasite development. However, these results suggest that a proper balance of gases somewhere near that in the gut may be one key to enhancing development of coccidia beyond that currently reported using standard *in vitro* culture conditions.

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ites each containing refractile bodies on either side of the nucleus, 2 DPI. 7. Nearly mature, giant second-generation meront, 3 DPI. 8. Mature, giant third-generation meront, 5 DPI. 9. Fourth-generation meront in degenerating host cell, 7 DPI.



## Relative Resistance of Brazil Strain Trypomastigote Forms of *Trypanosoma cruzi* to *In Vitro* Antibody-dependent Complement-Mediated Lysis

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**ABSTRACT:** Various assay conditions were employed in experiments examining the susceptibility of trypomastigote forms of the Brazil strain of *Trypanosoma cruzi* to antibody-dependent complement-mediated lysis. To identify optimal assay conditions, both guinea pig serum and normal human serum were used as complement sources, and fibroblast-derived or blood-form trypomastigotes were either coincubated with immune sera and complement together, or the parasites were first precoated with antibodies and then were incubated in complement. Under conditions promoting maximal lysis by antibodies and complement, 60-90% of the trypomastigote forms were not lysed. These results indicate that trypomastigotes of certain isolates of *T. cruzi*, such as the Brazil strain, may possess an escape mechanism by which they evade complement-mediated lysis.

Numerous studies have investigated the interaction of the complement system with *Trypanosoma cruzi*, the causative agent of Chagas' disease. Invertebrate stage epimastigotes of *T. cruzi* are susceptible to lysis by the alternative complement pathway (Muniz and Borriello, 1945; Nogueira et al., 1975). Recent studies by Sher et al. (1986) have indicated that as these forms differentiate to metacyclic trypomastigotes, the stage infective to vertebrate hosts, they express proteins on their surface that may block lysis by this pathway. Thus, resistance to lysis by the alternative complement pathway appears to be an adaptation these parasites have made to survive in the vertebrate host. Additional studies have demonstrated that the trypomastigote form of *T. cruzi* is susceptible to lysis by complement when bound by specific antibodies *in vitro*. For example, several reports have shown that trypomastigotes of the Y strain are highly susceptible to lysis by antibody and complement (Kierszenbaum, 1976; Krettli et al., 1979; Stefani et al., 1983; Romeiro et al., 1984; Kipnis et al., 1985). Additional reports have demonstrated that Tulahuen strain trypomastigotes (Budzko et al., 1975; Kierszenbaum, 1976) and metacyclic try-

omastigotes (Kierszenbaum and Lima, 1983) can likewise be killed.

However, the susceptibility of trypomastigote forms is not absolute and differences in the conditions required for lysis of different isolates of *T. cruzi* have been reported. CL and Gilmar strain trypomastigotes were more resistant to complement-mediated lysis than the Y strain (Krettli et al., 1979), and CL strain trypomastigotes incubated with specific antibodies were not agglutinated, and this treatment did not reduce their infectivity to mice (Krettli and Brener, 1976). Also, Y strain parasites were not lysed by human or murine IgM class parasite-specific antibodies and complement (Stefani et al., 1983; Romeiro et al., 1984) and, of the immunoglobulin classes, IgM is considered the most efficient in activating complement on the surface of cells. Thirdly, even with the susceptible parasite strains, a small percentage of organisms survives the treatment with antibody and complement. When combined, all of the above suggest that trypomastigote forms may possess a mechanism for escaping lysis, and that this activity may be greater in certain strains. Herein, we report that trypomastigotes of the Brazil strain of *T. cruzi* have a low susceptibility to complement-mediated lysis. Thus, our data provide evidence for the hypothesis that susceptibility to lysis varies with parasite strains.

In 1 of the 2 assay procedures that were used, antibodies and complement were incubated with Brazil strain parasites at the same time. For these assays, fibroblast-derived trypomastigotes (FDT's), no more than 24 hr old, were harvested from infected cultures of SVB6KH fibroblasts (Tarleton et al., 1983). To obtain blood-form trypomastigotes (BFT's), C3H(He) Dub mice were infected subcutaneously (s.c.) with 10<sup>5</sup> BFT's and then they were immunosuppressed (Stefani et al., 1983) by 2 intraperitoneal (i.p.) injections of cyclophosphamide (350 mg/kg body weight per injection), given on days 1 and 12 postinfect-

tion. These mice were bled by cardiac puncture on day 17, and BFT's were harvested for assays as described earlier (Hatcher and Kuhn, 1982). Epimastigote culture forms (CF's) were obtained from liver infusion tryptose medium cultures during the fourth passage of cultures initiated from blood of infected mice, as described earlier (Powell and Kuhn, 1980). Before use, parasites were washed 2 times by centrifugation in veronal buffer containing 10% gamma globulin-free fetal bovine serum (VB-FBS), which was also used as the diluent for sera and parasites in assays (Powell and Kuhn, 1980). Guinea pig serum (GPS), purchased from GIBCO (Grand Island, New York), or normal human serum (NHS) were used as complement sources. Immune serum (IS) was obtained from C57Bl/6 (B6), mice that were first infected with  $10^3$  Brazil strain BFT's, and then were given an injection of  $10^8$  CF's on day 90 postinfection. They were then bled on day 97. Additional B6 mice were infected i.p. with  $10^3$  BFT's and were bled during the acute phase of infection on day 28 (D28-S). Normal serum (NS) was obtained from uninfected age-matched B6 mice. Both infected and normal mice were bled by cardiac puncture while under ether anesthesia and the blood was allowed to clot, first at room temperature for 1 hr and then overnight at 4 C. Both NS and IS were sterilized through a 0.22- $\mu$ m filter, heat inactivated, and stored at -70 C before use. Assays were performed by combining 50  $\mu$ l of test serum,  $2.5 \times 10^5$  parasites in 50  $\mu$ l of VB-FBS, and 100  $\mu$ l of complement in plastic test tubes, and these were incubated in a water bath for 1 hr at 37 C. Each treatment was run in duplicate. To dilute the antibodies and complement prior to counting the parasites, 0.8 ml of VB-FBS was added to each tube which were centrifuged at 400 g for 7 min at 4 C. Then 0.8 ml was removed from each tube and 0.3 ml of fresh VB-FBS was added. The tubes were then placed in an ice bath while the numbers of motile parasites were counted using a hemocytometer.

When Brazil strain FDT's or BFT's were incubated with the IS and complement at the same time, the majority of the parasites was not lysed (Table I). Lysis of FDT's at the highest concentrations of IS was only 19.2% and 27.4% for the 1:2 and 1:40 dilutions of GPS, respectively. Much lower killing of FDT's by GPS was obtained using sera collected during the acute phase of infection. The day 28 sera yielded lysis of 4.2% and 13.6% for 1:2 and 1:40 dilutions of the complement source. Using NHS, the lysis obtained

at a final dilution of 1:4 of the IS was 23.3% and 6.2% for the 1:2 and 1:40 dilutions of this complement source. Because the greatest killing of FDT's in this assay was obtained using the 1:40 dilution of GPS, an experiment was performed in the same manner with BFT's and the extent of lysis detected was similar; only 23.9% of the BFT's were killed at the lowest dilution of the IS.

Antibody-dependent complement-mediated killing of FDT's was compared with that of CF's. Using a 1:40 dilution of GPS, the percentages of CF's killed by 1:4, 1:16, and 1:64 dilutions of IS were 11.8%, 80.3%, and 96.9%, respectively (Table I). In the same experiment, the highest killing of FDT's obtained was only about 12%. These results indicate that the assay conditions were valid to promote killing by the complement system. The lysis of CF's may have been greater at the 1:64 dilution of IS because this dilution provided optimal concentrations of antibody and complement. Alternatively, IgM class antibodies in the IS may have acted (in some manner) as blocking antibodies at the lower dilutions, since, as was indicated earlier, they apparently fail to direct complement-mediated lysis of *T. cruzi* (Stefani et al., 1983). In contrast to earlier reports (Muniz and Borriello, 1945; Nogueira et al., 1975; Sher et al., 1986), CF's in this experiment were not lysed through activity of the alternative complement pathway. No killing of CF's was obtained by treatment with complement alone (Table I). Two previous studies have reported the same finding (Anziano et al., 1972; Powell and Kuhn, 1980). Although additional experiments defining the requirements for lysis of CF's are required, we suggest this result may have been obtained because the CF's were from cultures recently established from the blood of infected mice (Powell and Kuhn, 1980).

Additional experiments were performed in which assay parameters were changed in an attempt to identify conditions in which a higher number of trypomastigotes were killed. Previous studies that have described nearly 100% killing of Y strain BFT's have used assays in which the parasites were first incubated with specific antibodies alone, and then complement was added (Stefani et al., 1983; Romeiro et al., 1984; Kipnis et al., 1985). Thus, for our assays,  $2 \times 10^5$  BFT's in 25  $\mu$ l of diluent and 25  $\mu$ l of IS were combined in wells of microtiter plates and they were incubated at 4 C for 1 hr. Next, 50  $\mu$ l of GPS was added to each well and the plates were incubated



TABLE I. Relative resistance of Brazil strain *Trypanosoma cruzi* fibroblast-derived trypomastigotes (FDT's) and blood-form trypomastigotes (BFT's) to antibody-dependent complement-mediated lysis when assayed by coin-cubation of sera with a complement source.

Exp. no.	Treatment†	Com- plement dilution	% Lysis*			Complement only
			1/final serum dilution			
			4	16	64	
1	FDT + IS + GPS	1:2	19.2 ± 4.8	5.7 ± 6.2	-6.7 ± 8.7	—
	FDT + D28-S + GPS	1:2	4.2 ± 10.0	-1.9 ± 2.7	8.4 ± 2.6	—
	FDT + NS + GPS	1:2	1.0 ± 1.2	0.9 ± 10.6	1.0 ± 18.5	—
	FDT + GPS	1:2	—	—	—	-10.9 ± 11.2
2	FDT + IS + GPS	1:40	27.4 ± 0.5	9.7 ± 3.3	1.1 ± 8.8	—
	FDT + D28-S + GPS	1:40	13.6 ± 10.0	8.1 ± 1.1	3.4 ± 4.4	—
	FDT + NS + GPS	1:40	-1.3 ± 13.3	0.1 ± 13.2	-2.0 ± 14.6	—
	FDT + GPS	1:40	—	—	—	-3.2 ± 10.2
3	FDT + IS + NHS	1:2	23.3 ± 14.2	13.2 ± 4.4	-3.9 ± 6.6	—
	FDT + NS + NHS	1:2	6.2 ± 7.6	ND‡	ND	—
	FDT + NHS	1:2	—	—	—	-2.4 ± 2.2
	FDT + IS + NHS	1:40	6.2 ± 12.0	11.6 ± 8.8	11.7 ± 0	—
	FDT + NS + NHS	1:40	-1.6 ± 5.4	ND	ND	—
	FDT + NHS	1:40	—	—	—	2.3 ± 6.6
4	BFT + IS + GPS	1:40	23.9 ± 4.9	16.9 ± 12.3	2.6 ± 10.0	—
	BFT + NS + GPS	1:40	1.8 ± 11.2	ND	ND	—
	BFT + GPS	1:40	—	—	—	-12 ± 0
5	FDT + IS + GPS	1:40	10.2 ± 9.0	11.9 ± 0	-3.8 ± 3.5	—
	FDT + NS + GPS	1:40	3.8 ± 3.5	1.3 ± 0	-15.2 ± 1.8	—
	FDT + GPS	1:40	—	—	—	6.4 ± 14.4
	CF + IS + GPS	1:40	11.8 ± 6.2	80.3 ± 3.7	96.9 ± 0	—
	CF + NS + GPS	1:40	10.1 ± 3.6	10.1 ± 6.0	0.2 ± 10.9	—
	CF + GPS	1:40	—	—	—	-17.7 ± 9.4

$$* \% \text{ Lysis} = 100 - \left( \frac{\text{treatment count}}{\text{buffer only}} \times 100 \right).$$

† For complete assay conditions, see text. Abbreviations: immune serum, IS; day 28 postinfection serum, D28-S; normal serum, NS; guinea pig serum, GPS; normal human serum, NHS.

‡ Not determined.

at 37 C for an additional hour. Finally, 100  $\mu$ l of diluent was added to each well, the plates were placed in an ice bath, and motile parasites were counted as above. Each sample was assayed in triplicate. BFT's were obtained as described above, except that infected and immunosuppressed A/J mice were used as donors. Three pools of IS, prepared from infected B6 mice, were used as sources of specific antibodies. IS-1 was obtained from B6 mice that had been infected i.p. with 10<sup>3</sup> BFT's 70–210 days previously. The mice were then given an injection of 10<sup>8</sup> CF's s.c. and were bled 7 days later. Pools of essentially identical sera designated IS-2 and IS-3 were collected from B6 mice that were first infected i.p. with 10<sup>3</sup> BFT's on day 0, and were injected s.c. with 10<sup>8</sup> CF's on days 75 and 90 postinfection. These mice were also bled 7 days after the third injection. In place of VB-FBS, Cedarlane Cytotoxicity Medium (Accurate Chemical and Scientific, Westbury, New York) was used as a diluent for both the sera and parasites. All other procedures were performed as described above.

Preincubation of Brazil strain BFT's with IS caused a higher number of parasites to be lysed, as was expected, but still the majority were not killed. At a 1:4 final dilution of the 3 IS, the lysis of BFT's obtained when incubated with a 1:2 dilution of GPS were 17.1%, 35.4%, and 40.1% (Table II). Thus, even at the highest level of killing, approximately 60% of the trypomastigotes survived. For the 1:40 dilution of GPS overall, fewer parasites were lysed; IS-1 and IS-2 yielded maximal lysis of 26.6% and 22.2%, respectively, at a 1:100 serum dilution, and the greatest lysis obtained with IS-3 was 21.3% at a dilution of 1:20 (Table II). As expected, no significant killing of BFT's was detected by incubation in the heat-inactivated IS, NS, or by incubation in NS with active complement. In contrast to the assay in which antibody, complement, and parasites were incubated together at the same time, incubating the parasites with antibody and then with complement yielded greater lysis at the lower complement dilution. This result is consistent with the observations of Krettli et al. (1979) who found that to obtain lysis of Y strain BFT's that were

TABLE II. Relative resistance of Brazil strain *Trypanosoma cruzi* BFT's to antibody-dependent complement-mediated lysis when assayed by preincubation of BFT's in sera followed by incubation with a complement source.

Exp. no.	Treatment†	Complement dilution	% Lysis*			
			1/final serum dilution			
			4	20	100	500
6	BFT + IS-1 + GPS	1:2	17.1 ± 6.1	16.2 ± 12.6	6.4 ± 11.9	3.7 ± 16.7
	BFT + IS-2 + GPS	1:2	35.4 ± 2.7	26.6 ± 7.5	14.1 ± 16.2	3.7 ± 5.5
	BFT + IS-3 + GPS	1:2	40.1 ± 10.8	18.5 ± 4.9	15.8 ± 14.2	2.0 ± 0
	BFT + NS + GPS	1:2	-6.1 ± 1.4	ND‡	ND	ND
	BFT + IS-1	—	2.3 ± 9.1	ND	ND	ND
	BFT + IS-2	—	2.3 ± 2.1	ND	ND	ND
	BFT + IS-3	—	6.7 ± 4.6	ND	ND	ND
	BFT + NS	—	-8.8 ± 9.8	ND	ND	ND
7	BFT + IS-1 + GPS	1:40	15.6 ± 12.6	13.8 ± 4.4	26.6 ± 9.6	6.3 ± 6.5
	BFT + IS-2 + GPS	1:40	9.4 ± 6.0	17.8 ± 4.3	22.2 ± 7.9	14.0 ± 19.5
	BFT + IS-3 + GPS	1:40	10.5 ± 8.3	21.3 ± 11.9	11.0 ± 5.5	19.4 ± 3.1
	BFT + NS + GPS	1:40	2.4 ± 5.7	ND	ND	ND
	BFT + NS	1:40	7.2 ± 8.9	ND	ND	ND

$$* \% \text{ Lysis} = 100 - \left( \frac{\text{treatment count}}{\text{complement only}} \times 100 \right).$$

† For complete assay conditions, see text. Abbreviations: immune serum, IS; normal serum, NS; guinea pig serum, GPS; normal human serum, NHS.

‡ Not determined.

precoated with antibodies, a high concentration of complement was required.

Results of the experiments described above indicate that the Brazil strain of *T. cruzi* has a low susceptibility to antibody-dependent complement-mediated lysis. This finding suggests that different isolates of *T. cruzi* vary in resistance to this effector component of the immune system, and that some strains, such as the Brazil, may have the means to subvert it. This putative evasive action could be directed against bound antibodies or involve inactivation of complement components. Previous studies have described capping, or shedding of surface-bound antibodies by trypomastigotes of *T. cruzi* (Schmunis et al., 1978; Krettl et al., 1979). We are currently performing experiments to determine if subpopulations of trypomastigotes can inactivate antibodies on their surfaces, and we plan to examine the interaction of Brazil strain trypomastigotes with complement components. This work was supported by Public Health Service Grant AI-13128 from the National Institutes of Health.

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## Indirect Immunofluorescent Detection of Oocysts of *Cryptosporidium parvum* in the Feces of Naturally Infected Raccoons (*Procyon lotor*)\*

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**ABSTRACT:** Fecal samples from 100 wild raccoons were examined for the presence of oocysts of *Cryptosporidium parvum* using a commercially available indirect immunofluorescent detection procedure. Thirteen (13%) of the samples were positive for oocysts. All positive samples were from juvenile raccoons. Over 61% of the infected samples contained moderate to large numbers of oocysts. Raccoons may serve as potential reservoirs for transmission of *C. parvum*.

Cryptosporidiosis caused by the coccidian parasite *Cryptosporidium parvum* is now recognized as an important cause of gastroenteritis and diarrhea in a number of mammalian species, including man (Current, 1985; Fayer and Ungar, 1986; Moore et al., 1988). It has become especially important as a cause of severe, protracted diarrhea in the immunocompromised human patient (Soave and Armstrong, 1986).

Numerous methods have been described to directly detect oocysts in the feces of the infected host (Current, 1985; Fayer and Ungar, 1986; Moore et al., 1988). However, due to their small size and paucity in some samples, they may be

overlooked. Oocysts of this parasite may also be confused with yeast cells. The use of fluorescent microscopy in conjunction with specific immunological probes to detect oocysts in fecal matter has been shown to be a highly sensitive and specific method (Stibbs and Ongerth, 1986; Garcia et al., 1987).

The apparent lack of host specificity complicates the epidemiology of this parasite. The role of domesticated livestock, companion animals, and wildlife in the transmission of this parasite to humans or other mammalian species has been suggested (Carlson and Nielsen, 1982; Current, 1985; Klesius et al., 1986). The raccoon (*Procyon lotor*) is one particular wildlife species that is found commonly in both urban and rural environments. For this reason and because of its potential to transmit this parasite to other mammalian hosts, a study was undertaken to determine the prevalence of oocysts of *C. parvum* in the feces of naturally infected raccoons from central Illinois.

One hundred rectal fecal samples were obtained in December 1987 from hunter-shot or trapped raccoons in central Illinois. Information concerning the collection of specimens from these animals has been described previously (Snyder and Fitzgerald, 1987). Approximately 5 g of feces

\* Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

from each animal were added to 20 ml of water, mixed thoroughly, and this suspension used to fill 15-ml plastic conical centrifuge tubes. Each sample was centrifuged for 6 min at 1,500 g, the supernatant fluid discarded, the tube filled with Sheather's sugar solution, mixed thoroughly, and centrifuged again as just described. The contents from the top half of each centrifuge tube were withdrawn, placed in a new 15-ml screw-capped glass centrifuge tube, and washed 3 times by centrifugation with 0.1 M phosphate-buffered saline (pH 7.6). After the third wash, the supernatant fluid was discarded and the pellet representing oocysts concentrated from 5 g of feces was re-suspended in 5 ml of 10% neutral buffered formalin. All samples were stored at 4 C until examined.

Each fecal sample was subjected to the commercially available Merifluor® *Cryptosporidium* indirect immunofluorescent (IIF) detection procedure (Meridian Diagnostics, Inc., 3471 River Hills Drive, Cincinnati, Ohio 45244). This procedure for the detection of oocysts of *Cryptosporidium* sp. in fecal matter and other environmental sources uses a monoclonal anti-cryptosporidial antibody that binds to the wall of the oocyst. This reaction is visualized by the addition of a fluorescein isothiocyanate-conjugated anti-immunoglobulin. The resulting apple green fluorescence, visualized with the aid of a fluorescent microscope, and characteristic structure (4–5  $\mu$ m in size) indicate the presence of oocysts and constitute a positive test result. Positive and negative controls are provided for each usage. The detection procedure was followed as per the instructions provided with the test kit, using concentrated preserved oocysts from the feces of raccoons as described above.

Thirteen (13%) of the 100 fecal samples were positive for oocysts of *C. parvum* using the IIF procedure. All positive samples were from juvenile raccoons. The numbers of oocysts present in each positive sample were arbitrarily classified as either small ( $\leq 5$  oocysts per 100 $\times$  ocular field), moderate ( $\geq 6$  to  $\leq 50$  oocysts per 100 $\times$  ocular field), or large ( $\geq 51$  oocysts per 100 $\times$  ocular field). Of the 13 positive samples, 5, 4, and 4 were classified as having small, moderate, or large numbers of oocysts, respectively. All of the oocysts observed had a bright apple green fluorescence outlining the oocyst wall and there was no difficulty in interpreting positive oocysts. Eggs of *Baylisascaris procyonis*, *Physaloptera rara*, *Ar-*

*throcephalus lotoris*, and *Capillaria* spp. and oocysts of *Eimeria* spp. were routinely seen in samples, but were never seen to exhibit any fluorescence. Yeast cells and other fecal flora (unidentified) also did not exhibit any fluorescence. The spores of *Monocystis* sp., a gregarine of earthworms present in several fecal samples, did show a slight outline of fluorescence; however, the characteristic shape of these spores can be distinguished easily from oocysts of *C. parvum*.

The IIF procedure as described above appears to be highly specific and it clearly detected oocysts of *C. parvum* in fecal samples obtained from wild raccoons. This IIF procedure has also proven useful and highly specific at this laboratory (unpubl. results) as a routine screen to detect oocysts of *C. parvum* in calves with diarrhetic stools. The fecal samples were concentrated in this study using Sheather's sugar solution; this concentration procedure is important if small numbers of oocysts are present. This is exemplified in this study by the fact that 5 of the 13 positive samples yielded only small numbers of oocysts. Comparisons between the various staining methods used to detect oocysts of *C. parvum* have been reported (Henriksen and Pohlenz, 1981; Garcia et al., 1983; Casemore et al., 1985). The use of monoclonal antibody probes in conjunction with a fluorescent label has proven to be the most sensitive technique, 10 times more sensitive in one study, particularly when small numbers of oocysts are present in the sample (Garcia et al., 1987). Acid-fast methods do not always stain all oocysts consistently in a sample. Thus, the increased sensitivity of the monoclonal antibody procedure provides an ideal screening method (Pohjola et al., 1985).

The zoonotic potential of raccoons acting as reservoirs for transmission of infection to other mammals has not been documented previously. The wide distribution and varied habitats of raccoons make them an ideal source of contamination to other mammalian species. Humans, domestic livestock, companion animals, and other wildlife such as those found in nature, zoos, and wildlife parks may be routinely exposed to oocysts of *C. parvum* of raccoon origin. Individuals living or working in close association with infected raccoons may be at a greater risk of exposure. Immunocompromised mammals should not be exposed to species of wildlife, such as the raccoon, so as to reduce the risk of exposure to this parasite.



The pathogenicity of isolates of *C. parvum* of raccoon origin to other mammalian species, including raccoons, is not known. It has been suggested that certain isolates of *Cryptosporidium* may differ greatly in their virulence (Current, 1985). A previous report of *Cryptosporidium* from a raccoon diagnosed through histopathology indicated that the animal appeared healthy prior to euthanasia (Carlson and Nielsen, 1982). Opossums, experimentally inoculated with a calf isolate of *C. parvum*, appeared to be affected only mildly (Lindsay et al., 1988). Additional information is needed on the infectivity of wildlife isolates of *C. parvum* and their potential as reservoirs for transmission of infection to domestic animals and man.

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## Glutamine and Asparagine Synthesis in the Nematodes *Heligmosomoides polygyrus* and *Panagrellus redivivus*

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**ABSTRACT:** Low levels of glutamine synthetase were demonstrated in *Panagrellus redivivus* and *Heligmosomoides polygyrus*. Asparagine synthetase was detected in *P. redivivus* but activity was too low to be detected in *H. polygyrus*.

Parasitic helminths are capable of rapid growth and characteristically produce large numbers of

eggs or larvae. Synthetic reactions would, therefore, appear to be potential sites for chemotherapy. Despite this there have been few studies on amino acid metabolism in helminths. Both glutamine and asparagine have been identified in protein hydrolysates from a range of helminths (Von Brand, 1973). In addition, *Fasciola hepatica* (Locatelli and Camerini, 1969), *Hymenolepis*

TABLE I. Glutamine synthetase and asparagine synthetase activity in *Heligmosomoides polygyrus* and *Panagrellus redivivus*.

Enzyme	Activity (nmoles hydroxamate formed per min per mg protein, at 30 C; mean $\pm$ SEM, n = 10)		
	<i>H. polygyrus</i>	<i>P. redivivus</i>	Rat liver
Glutamine synthetase	0.25 $\pm$ 0.01	0.05 $\pm$ 0.03	0.77 $\pm$ 0.44
Asparagine synthetase	0*	0.06 $\pm$ 0.01	0.07 $\pm$ 0.04

\* <0.01 nmoles/min/mg.

*diminuta* (Zavras and Roberts, 1984), and a number of plant-parasitic nematodes (Myers and Krusberg, 1965) have been reported to excrete glutamine and asparagine during *in vitro* incubations. The incorporation of radio-labelled precursors into these 2 amino acids has been demonstrated in 2 helminths, *Bipalium kewense* (Campbell, 1965) and *Moniliformis moniliformis* (Bryant and Nicholas, 1965), but there have been no studies on the enzyme systems involved.

The activities of asparagine and glutamine synthetases in the parasitic nematode *H. polygyrus* and the free-living nematode *P. redivivus* are shown in Table I; the activity in rat liver is included for comparison. Adult female *H. polygyrus* were isolated from infected mice as previously described (Grantham and Barrett, 1986) and *P. redivivus* were obtained from oatmeal cultures (Barrett and Butterworth, 1984). The assay for glutamine synthetase (E.C. 6.3.1.2) was modified from Rowe et al. (1970). The assay mixture contained (in a final volume of 1 ml): imidazole-HCl buffer, pH 7.2, 50  $\mu$ moles; manganese chloride, 20  $\mu$ moles; mercaptoethanol, 25  $\mu$ moles, L-glutamate, 20  $\mu$ moles, ATP, 10  $\mu$ moles; hydroxylamine, 100  $\mu$ moles. After incubation for 2 hr at 30 C the reaction was stopped by the addition of 0.25 ml of 0.5 M trichloroacetic acid and the solution centrifuged at 150 g to remove the denatured protein. The 4-glutamylhydroxamate formed was determined with the ferric chloride reagent (Hill, 1947). Asparagine synthetase activity (E.C. 6.3.1.1) was determined by the method of Ravel (1970). The assay mixture contained (in a final volume of 1 ml): Tris-HCl buffer, pH 6.4, 50  $\mu$ moles; manganese chloride, 20  $\mu$ moles; mercaptoethanol, 25  $\mu$ moles; L-aspartate, 50  $\mu$ moles; ATP, 10  $\mu$ moles; hydroxylamine, 100  $\mu$ moles. The 3-aspartylhydroxamate formed was determined as above.

Low levels of glutamine synthetase were found in both nematodes, but asparagine synthetase activity was too low to be detected in *H. polygyrus*. As well as being an important constituent of proteins, glutamine has a central role in the nontoxic transfer of ammonia within the tissues as well as being an amino group donor in a wide range of synthetic reactions.

Although amino acids are not a significant energy source in parasitic helminths there is now increasing evidence that helminths possess most, if not all, of the enzymes required for the synthesis and degradation of amino acids.

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## Isoelectric Focusing of Ten Strains of *Giardia duodenalis*

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**ABSTRACT:** Ten strains of human- and animal-source *Giardia duodenalis* were evaluated using an isoelectric focusing technique. Banding patterns obtained from total cell proteins of trophozoites demonstrated both similarities and differences between strains. This confirms the heterogeneity of this morphological group of *Giardia* sp. demonstrated by others. Heterogeneity was demonstrated among the strains retrieved from human and animal hosts and from hosts within the same geographical region.

*Giardia* trophozoites have been classified into 3 groups on the basis of morphological criteria (Filice, 1952). Trophozoites of the *G. agilis* group, infecting amphibians, have not been cultured *in vitro*. A single report of apparently successful *in vitro* cultivation of *G. muris* has been reported (Gonzalez-Castro et al., 1986). Techniques for the *in vitro* propagation of *G. duodenalis* (including *G. lamblia*) are well described (Keister, 1983; Meyer and Radulescu, 1984). The first human-source *G. duodenalis* was cultured axenically in 1976 (Meyer, 1976) and most strains successfully retrieved to date are still human-source. Studies analysing different strains of *G. duodenalis*, including techniques of isoenzyme analysis (Bertram et al., 1983; Meloni et al., 1988), endonuclease restriction analysis of DNA (Nash et al., 1985), and response to *in vitro* excystation and culture techniques (Meloni and Thompson, 1987), suggest that intraspecific variation occurs within this morphologic group. Host specificity, however, appears to be unreliable as a criterion for classifying *Giardia* trophozoites (Meyer, 1985). Consideration of isolate differences also appears important in the pathogenesis of infection. Two recent studies (Aggarwal and Nash, 1987; Visvesvara et al., 1988) demonstrated that human-source *Giardia* isolates produce different patterns of infection and immune responses in their gerbil hosts. In the present study, we report differences in banding patterns obtained by isoelectric focusing of trophozoite protein from 10 human- and animal-source isolates.

Five recently axenized strains of *G. duodenalis*

were included in this study (Isaac-Renton et al., 1986). Three of these isolates were obtained from persons who acquired their infection in the western Canadian province of British Columbia and 2 were from beavers trapped at different sites in the province. Five other strains of the *G. duodenalis* group (kindly provided by Dr. G. Faubert, Institute of Parasitology, McGill University, Montreal, Canada), including 2 beaver strains from the contiguous province of Alberta, were also tested. Isoelectric focusing was carried out using lysate preparations of trophozoites from each *Giardia* strain. Trophozoites were cultured in TYI-S-33 medium (Keister, 1983) at 35 C without antibiotics. Subculture of all test preparations, using standard methods to detect bacterial or fungal growth, was carried out prior to and after lysate preparation. Test samples were prepared by harvesting 72-hr-old cultures by chilling in ice water for 10 min, centrifuging at 475 g for 15 min, and washing sediments 3 times in sterile phosphate-buffered saline, pH 7.2. Trophozoites, resuspended in an equal volume of a solution of 2 mM EDTA, 2 mM dithiothreitol, and 2 mM  $\alpha$ -caproic acid, were then disrupted by freeze-thawing and lysates centrifuged at 4,700 g for 30 min. Supernatants were aliquoted and frozen at -70 C until use.

A 1-mm agarose gel was prepared using 0.3 g Agarose IEF (Pharmacia Fine Chemicals, Uppsala, Sweden), 3.6 g sorbitol (Difco Laboratories), and carrier ampholytes (Pharmalytes, pH range 3–10, Pharmacia Fine Chemicals). Each test sample (20–25  $\mu$ g protein: Bio-Rad System of protein determination, Bio-Rad Laboratories, Richmond, California) and a series of characterized pI protein markers (Broad pI protein markers, Broad pI Calibration Kit, Pharmacia Fine Chemicals) were applied in duplicate on each gel and a minimum of 5 samples for each isolate was electrophoresed on different gels using an FBE 3000 flat-bed electrophoresis apparatus (constant power of 15 watts, maximum voltage of 1,500, current unlimited). Gels were stained (0.2%

Coomassie blue R 250), destained until the background was clear (35% ethanol and 10% acetic acid), and dried. Standard curves were calculated using the known pI values for each gel.

No differences in banding patterns were noted between duplicate samples on the same gel or between samples from the same isolate on different gels. As expected, many protein bands were demonstrated on each isolate but differences in banding patterns between isolates were also clearly seen. For example, as noted in Figure 1, lysates from some isolates (lanes 1, 2, and 5) produced an extra doublet of bands present at pI 8.2 and not noted in the patterns from other isolates. On comparing the patterns obtained from the human-source British Columbia isolates (lanes 1, 2, and 3), differences were noted. The patterns obtained from the British Columbia beaver-source isolates (lanes 4 and 5) also appeared different from each other. The 4 beaver isolates, including 2 strains from Alberta, Canada (lanes 4, 5, 6, and 7), produced banding patterns that appeared different. As reported in a

previous study (Nash et al., 1985), one Alberta beaver-source isolate (Be-1) appeared dissimilar to the other Alberta beaver-source isolate (Be-2), as well as WB and the Portland-1 strains. In this study, Be-1 (lane 6) was also dissimilar to all 3 other beaver isolates but appeared similar to one (lane 3) of the British Columbia human-source isolates.

This study further confirms the heterogeneity that occurs among isolates of the *G. duodenalis* morphologic group. Although this rapid, simple technique clearly demonstrates differences between isolates, it will probably not be useful in characterizing strains for epidemiologic or taxonomic purposes if total cell proteins are analysed. To overcome the complexity of the banding patterns of total cell proteins, comparison of defined trophozoite proteins may be more useful. As demonstrated in other work (Nash et al., 1985; Meloni et al., 1988), this study also shows that neither geographical location nor the nature of the host was predictable when strains, retrieved from hosts residing in a limited geographical area

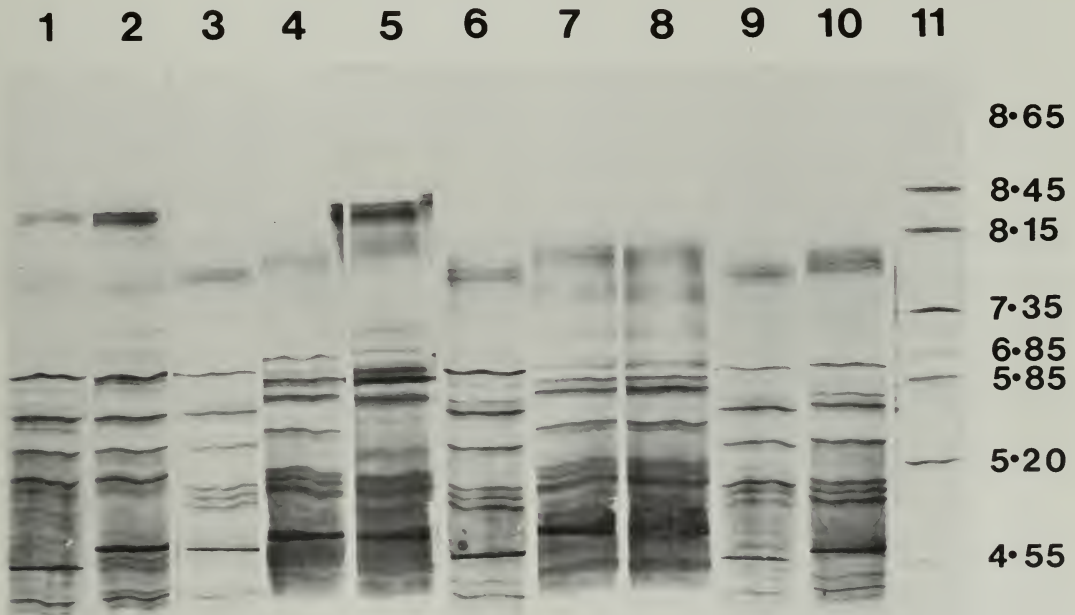


FIGURE 1. Isoelectric focusing patterns of lysates of 10 strains of *Giardia duodenalis* trophozoites. Lane 1, British Columbia human-source isolate; lane 2, British Columbia human-source isolate; lane 3, British Columbia human-source isolate; lane 4, British Columbia beaver-source isolate; lane 5, British Columbia beaver-source isolate; lane 6, Alberta beaver-source isolate (Be-1); lane 7, Alberta beaver-source isolate (Be-2); lane 8, dog-source isolate; lane 9, WB strain (ATCC 30957); lane 10, Portland-1 strain (ATCC 30888); lane 11, proteins with known pI values. The pI values are on the right side of the figure.



from both human and animal sources, were compared.

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## Interaction of Bass Tapeworm, *Proteocephalus ambloplitis*, and *Neoechinorhynchus* sp. (Acanthocephala) in Largemouth Bass, *Micropterus salmoides*

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**ABSTRACT:** The number of plerocercoids of the bass tapeworm, *Proteocephalus ambloplitis*, in wild largemouth bass was negatively correlated ( $r = -0.94$ ) with the number of *Neoechinorhynchus* sp. Competitive inhibition between the 2 parasites appeared to exist. Similarly, the numbers of *Neoechinorhynchus* sp. in wild bass decreased when adult bass tapeworms were present in the intestine. *Proteocephalus ambloplitis* plerocercoids used to challenge bass vaccinated with either *P. ambloplitis* adult or *Neoechinorhynchus* sp. antigens were smaller ( $P < 0.05$ ) when recovered than those used to challenge control bass. Based on preliminary results, both antigens might have enabled the bass to limit growth and/or development of the invading bass

tapeworm, plerocercoids. Cross-protective immunity may be the reason for this occurrence, in which case, it could offer an explanation for competitive inhibition existing between *P. ambloplitis* and *Neoechinorhynchus* sp.

Competitive inhibition between cestodes and acanthocephalans has been observed in several fish species. Of 258 sticklebacks (*Gasterosteus aculeatus*) infected with *Proteocephalus filicollis* and/or *Neoechinorhynchus rutili*, only 24 were concurrently infected with both parasites (Chap-

TABLE I. Numbers of plerocercoids of bass tapeworms (*Proteocephalus ambloplitis*) in wild largemouth bass relative to numbers of the acanthocephalan *Neoechinorhynchus* sp. Correlations ( $r$ ) of the numbers present of both parasites.

Limits of infection	Number of fish	Average number of tapeworm plerocercoids	Average number of <i>Neoechinorhynchus</i> sp.	$r$
4-11 plerocercoids	2	7.50	5.50	-0.94
1-2 plerocercoids	4	1.25	13.50	
0 plerocercoids	16	0	21.13	

pel, 1969). Likewise, acanthocephalans were usually absent from the intestine of pike (*Esox lucius*) severely infected with cestode *Triaenophorus nodulosus* (Sheuring, 1923, from Dogiel, 1966), and in Siberia, least ciscoes (*Coregonus sardinella*) were never simultaneously infected with cestodes and acanthocephalans (Petrushevski, 1955, from Dogiel, 1966). In the present study, wild largemouth bass (*Micropterus salmoides*) were examined for numbers of naturally occurring bass tapeworms, *Proteocephalus ambloplitis* and *Neoechinorhynchus* sp. A preliminary investigation was also performed to determine if the mechanism mediating the competitive inhibition between these 2 parasites may have been host-mediated cross-protective immunity.

Twenty-two largemouth bass captured in Alabama and Georgia in 1982 and 1984 were dissected to determine the number of *P. ambloplitis* plerocercoids in the body cavity and *Neoechinorhynchus* sp. adults in the intestine (Durborow, 1986). No adult *P. ambloplitis* were present in these bass. In addition, numbers of *Neoechinorhynchus* sp. versus adult bass tapeworms were compared in 6 largemouth bass captured in 1968 (Durborow, 1986). No plerocercoid tapeworms were present in these 6 bass.

Much larger numbers of *Neoechinorhynchus* sp. were found in bass harboring no plerocercoids (Table I). As the number of plerocercoids in bass decreased, the number of *Neoechinorhynchus* sp. increased. These averages had a high negative correlation of -0.94.

Of the 6 bass collected in 1968, 3 had no intestinal adult tapeworms and 3 harbored 1 or 2 adult bass tapeworms in their intestines. The numbers of *Neoechinorhynchus* sp. recovered from the intestines of the 3 bass having no simultaneous adult tapeworm infection were 89,

TABLE II. Number and length of bass tapeworm plerocercoids recovered after challenge of 3 vaccinated and 3 control largemouth bass.

Bass number and vaccination treatment	Number of plerocercoids recovered out of 10 used in challenging	Mean length (mm) of plerocercoids by treatment	Mean length (mm) of plerocercoids (vaccination vs. control)
1. <i>Neoechinorhynchus</i>	5	10	11
2. <i>Neoechinorhynchus</i>	4	12	
3. <i>Proteocephalus ambloplitis</i>	10	12	
4. Control	6	23	20
5. Control	8	21	
6. Control	6	15	

61, and 49, whereas *Neoechinorhynchus* sp. recovered from the 3 adult tapeworm-infected bass numbered 26, 29, and 16, about one-third the number recovered from bass free of adult tapeworms.

To determine whether these 2 examples of competitive inhibition might have been due to host-mediated cross-protective immunity, bass were immunized with either *P. ambloplitis* or *Neoechinorhynchus* sp. antigen and were compared in their ability to resist a challenge of *P. ambloplitis* plerocercoids using procedures described in Durborow (1986). Controls receiving no immunizing injection were also challenged. Two bass receiving *Neoechinorhynchus* sp. soluble antigen and 1 bass receiving adult *P. ambloplitis* particulate antigen were immunized 3 times by ip injection at weekly intervals. These bass and 3 control bass were each challenged with 10 plerocercoids 20 wk after the last injection. The number of experimental bass was small due to the difficulty in finding a sufficient source of bass tapeworm plerocercoids to use for challenging. The interval between the challenge and necropsy of all 6 bass was 25 days. Numbers and lengths of plerocercoids recovered from all bass were recorded (Table II). The statistical significance of differences in numbers and lengths of recovered tapeworms was determined by  $t$ -tests; the 2 treatments were compared with each other and with controls.

The numbers of plerocercoids recovered did not differ among the treatments or the controls ( $P > 0.1$ ) (Table II). There was, however, a significant difference ( $P < 0.05$ ) in the lengths of plerocercoids recovered when comparing either treatment with the controls. Additionally, damaged plerocercoids were recovered from both *P.*



*ambloplitis*- and *Neoechinorhynchus* sp.-treated bass. These plerocercoids appeared to have been under attack by the immunized host.

These results are consistent with the lack of growth and maturation of the tapeworm *Caryophyllaeus laticeps* in dace (*Leuciscus leuciscus*) previously exposed to this tapeworm (Kennedy and Walker, 1969), and are consistent with the findings of Holmes (1961) where concurrent infections of *Hymenolepis diminuta* and the acanthocephalan *Moniliformis dubius* in rats caused size reduction in the cestode and slight size reduction in the acanthocephalan. Similarly, growth and maturation of the tapeworms *Vampirolepis nana* (Ito, 1977), *H. microstoma* (Howard, 1976), and *H. diminuta* (Heyneman, 1962; Hopkins et al., 1972, 1977; Elowni, 1982; Hopkins, 1982) were inhibited when homologous challenges were made in immunized mice, and in some cases destrobilation of the worms was observed (Hopkins et al., 1972; Hopkins, 1982). The weight and volume of *H. diminuta* were likewise reduced when this tapeworm was used to challenge rats immunized against a closely related *Hymenolepis* species (Heyneman, 1962; Schad, 1966).

Damage done to the bass tapeworm plerocercoids observed in the present study was also observed in metacestodes of the rat parasite, *Taenia taeniaeformis*, when exposed to rat serum (Conder et al., 1983). In addition, heavy connective tissue found surrounding the damaged tapeworm plerocercoids in this study was similar to the encapsulation of *Spirometra mansonoides* larvae in monkeys that had been injected with homogenates of adult *S. mansonoides* (Barriga, 1981). Barriga (1981) stated that there is ample proof that the tegument of cestodes can be destroyed by an immune response involving antibodies and complement. The possibility exists that antibodies and complement may have been responsible for the tegument damage found in the present study.

Host-mediated cross-protective immunity may have been the mechanism causing a high negative correlation between numbers of *P. ambloplitis* and *Neoechinorhynchus* sp. found in wild bass. This mechanism has been observed among protozoan parasites in channel catfish and rainbow trout: injections of *Tetrahymena pyriformis* cilia protected channel catfish against *Ichthyophthirius multifiliis* better than homologous vaccinations (Goven et al., 1980); similarly, 4-hr baths of *T. thermophila* cells or sheared cilia pro-

tected rainbow trout against *I. multifiliis* and *Ichthyobodo necator* (Wolf and Markiw, 1982).

The phenomenon of one parasite causing the host to reduce the numbers of another competing parasite species seems to occur in degrees where a low number of parasite *A* will reduce slightly the number of parasite *B* in the host, and a high number of parasite *A* will reduce the number of parasite *B* even more or eliminate them. This "antigenic threshold" phenomenon seemed to occur in this study (Table I). A similar occurrence was observed in rejection of tapeworm *H. citelli* by mice, where rejection was quicker and more complete in mice harboring 6 tapeworms versus mice with 1 worm (Hopkins and Stallard, 1974). In another study, mice initially infected with 30 or 6 *H. diminuta* rejected 91% and 76%, respectively, of the worms used in the challenge, although those initially infected with only 1 worm showed no significant rejection (Elowni, 1982). Quantitatively, 1 µg of either somatic or secreted products of *Taenia taeniaeformis* injected into rats provided 70% protection against homologous challenge, whereas 10 µg gave 90% protection and 50 µg conferred 100% protection (Kwa and Liew, 1977). Holmes' (1973) statement that immunity against parasites acts to regulate their populations, not to eliminate them completely, offers an explanation for the "antigenic threshold" phenomenon.

In conclusion, the competitive inhibition between *P. ambloplitis* and *Neoechinorhynchus* sp. in bass may be due to host-mediated cross-protective immunity as evidenced by size reduction and damage of *P. ambloplitis* plerocercoids used to challenge bass vaccinated with either *Neoechinorhynchus* sp. or *P. ambloplitis* antigens.

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## Behavior of *Leishmania braziliensis* s.l. in Golden Hamsters: Evolution of the Infection Under Different Experimental Conditions

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**ABSTRACT:** Reproducibility of *Leishmania braziliensis* s.l. metastatic behavior in hamsters was studied for 9 isolates of *L. b. panamensis* and 2 of *L. b. guyanensis* with a previous record of metastasis. Also, the influence of corticosteroids and trauma was evaluated. In the corticosteroid-treated group, metastases appeared earlier than in the nontreated group, and localization at the site of trauma was more frequent (4/9) than in the nontreated hamsters (1/5). Nine of the 11 strains (82%) were capable of reproducing metastatic behavior. Studies on dissemination of *L. b. panamensis* showed that the regional lymph node is invaded as

soon as 5 days postinfection, with further nonhematogenous dissemination to other tissues and organs in less than 4 wk.

A number of rodents and primates are susceptible to *Leishmania* spp. (Chang and Hendricks, 1985). Despite this, research on strains of the *Leishmania braziliensis* complex has been hampered by the unavailability of adequate experimental animal models of infection and disease.



TABLE I. *Experimental infection of hamsters with metastasizing strains of L. braziliensis (s.l.).*

	Hamsters with <i>M</i> */hamsters inoculated	$\bar{x}$ prepatent period (mo)†	Origin of inoculum		<i>M</i> at the site of trauma/hamsters with <i>M</i>
			Culture	<i>M</i>	
Group treated with corticosteroids	9/18	5.7	5	4	4/9
Nontreated group	5/18	7.1	2	3	1/5

\* *M* = metastasis.

† Period between primary infection and appearance of skin metastasis.

We obtained reproducible patterns of pathogenesis with subspecies and individual strains of *L. braziliensis*, based on the observation of golden hamsters inoculated with clinical samples. The qualitative and quantitative manifestations of disease seen may provide experimental access to host and parasite variables in disease expression that could reveal mechanisms of pathogenesis in *L. braziliensis* infection.

#### Metastatic behavior of *L. braziliensis s.l.*

Strains of *L. b. guyanensis* metastasize to the skin and viscera of infected hamsters (Lainson and Shaw, 1979). This has also been observed for *L. b. braziliensis* (Brazil, 1976; Wilson and Lollini, 1980). In one case (Wilson and Lollini, 1980) it was suggested that an impaired immune response might underlie metastatic spread.

In our laboratory, hamsters inoculated with triturated tissues and lesion aspirates of infected patients for diagnostic purposes are maintained for 12–18 mo. Weekly macroscopic observation of the infected hamsters initially revealed that 8 of 131 (approx. 6%) leishmanial strains inoculated produced cutaneous metastases (CM). Subsequently, nine (8.6%) of 105 *L. b. panamensis* strains showed CM between the sixth and 15th months postinoculation (PI), whereas 2 of 9 *L. b. guyanensis* strains produced CM between the 12th and 14th months PI. None of 18 *L. b. braziliensis* strains displayed this behavior. Metastases were usually localized in the toes and less frequently in the tail. Other tissues and organs were virtually free of lesions, except for the ears, in which small nodules were occasionally observed.

Based on these observations, 9 isolates of *L. b. panamensis* and 2 of *L. b. guyanensis* that had demonstrated CM were selected to determine whether metastasis was a reproducible characteristic of these strains. Each strain was inoculated subcutaneously in the snout of 2 animals with  $2 \times 10^5$  promastigotes harvested from Se-

nekje's medium at the end of the logarithmic phase of growth (culture day 6). The availability of hamsters infected with 7 of the 11 strains enabled us to run parallel experiments with lesion aspirates and cultured promastigotes. For this, 2 animals were inoculated with each of the 7 strains. One of each pair of hamsters inoculated with either cultured promastigotes or tissue aspirates were treated with corticosteroids (hydrocortisone, sodium succinate, UpJohn Co., 200 mg/kg/wk) for 6 mo. The external toe of the right hind foot was amputated on the 17th week PI to evaluate the influence of trauma on the appearance of metastasis.

The hamsters with metastases were sacrificed. Samples of popliteal lymph nodes, spleen, liver, bone marrow, and lymph nodes draining the head were cultured in Senekje's medium. The experiment was terminated on the 10th month of observation, when all the surviving animals were sacrificed.

Macroscopically, the type of lesions developed by the hamsters receiving corticosteroid treatment (CT) was similar to those not treated (NT). Moreover, the proportion of hamsters showing metastases not related to the site of experimental trauma was similar in both groups (5/18 in CT vs. 4/18 in NT). The mean time of appearance of these metastases was shorter in the CT group than in the NT group (Table I). The origin of the inoculum (culture or lesion aspirate) did not appear to influence the capacity to produce CM or the time of CM appearance (Table I).

The results of experimental trauma were clearly different in the CT group and the NT group. In the NT group, 1 of 5 (20%) metastases was localized at the site of trauma, whereas 4 of 9 (45%) metastases appeared at the site of the amputated toe in CT hamsters (Table I). As the prepatent period was similar for both spontaneous and posttraumatic metastases (data not shown), it is not clear if trauma could stimulate

TABLE II. Dissemination of *L. braziliensis panamensis* in experimentally infected hamsters, detected by culture in Senekjie's medium.

Days PI	Inoculum (ps $\times 10^6$ )*	No. of hamsters examined	No. of infected tissues or organs							
			Bone marrow	Head LN	Popliteal LN	CL popliteal LN	Snout†	Foot†	Spleen	Liver
5	5	3	0	3	3	0	3	3	0	0
30	10	3	3	3	3	2	3	ND	2	3

\* ps = parasites; LN = lymph node; CL = contralateral; ND = not done.

† Tissue of the inoculation site.

the appearance of the metastasis, but it evidently conditioned the process with respect to its localization in the hamster.

Although our record for metastases in hamsters used in diagnosis was 8 (6%) for 131 different strains, the reinoculation of 11 strains with a previous history of metastasis demonstrated 82% (9/11) reproducibility of this biological behavior. Thus, CM in this model may be a biological trait of certain *L. braziliensis* strains.

#### Dissemination of *L. braziliensis panamensis*

The biological events allowing amastigotes to survive and move from the inoculation site to distant tissues and organs of the host are ill-defined. Knowing the timing and sequence of events by which the parasite colonizes different host tissues is of practical importance, i.e., in evaluating topical and systemic treatments and whether selective concentration of the drug in certain tissues would be desirable.

For this, we undertook 2 pilot experiments to determine the time course in which amastigotes invade other host tissues, and whether blood transport could be one of the major mechanisms of distributing the parasite throughout the body.

Groups of 3 hamsters were inoculated subcutaneously in the snout and hind foot with  $5 \times 10^6$  stationary phase (culture day 6) promastigotes of *L. b. panamensis*. The animals were sacrificed 5 days PI, and samples of the inoculation site, lymph nodes, and viscera (see Table II) were cultured in 96-well flat-bottom microplates containing Senekjie's medium. At this time leishmaniae (Table II) were only present at the inoculation site and in the draining lymph nodes.

In an attempt to detect possible dissemination of *L. b. panamensis* through the blood, 3 hamsters were inoculated with  $10^7$  stationary phase promastigotes in the snout and hind foot.

Beginning 4 days after infection and every 4 days thereafter, hamsters were bled from the ret-

ro-orbital plexus to prepare Giemsa-stained smears and for culture in Senekjie's medium. The experiment was terminated 30 days PI and samples of lymph nodes, spleen, liver, and bone marrow were studied parasitologically. No parasites were observed in the blood smears within or outside of phagocytic white cells, and cultures seeded with whole blood or blood diluted 1:5 with PBS were all negative.

At the end of the experiment the cultures of the lymph nodes (including the contralateral ones), spleen, liver, and bone marrow were all positive, showing that by 30 days there was complete dissemination of *L. b. panamensis* (Table II). Thus, it appears that *L. b. panamensis* is immediately localized in the regional lymph node, with further nonhematic dissemination within the host. If parasitemias do occur, they are lower than 10 parasites per 0.05 ml, because our culture methods readily detect between 10 and 20 promastigotes.

Our experiments show that some strains of *L. b. guyanensis* and *L. b. panamensis* produce cutaneous metastasis in the hamster. Subsequent to the rapid localization of parasites in the draining lymph node, spread to other sites can occur in less than 4 wk. Early infection of the hamster's lymphatic system, followed by the invasion of other tissues possibly mimics and helps to explain the pathogenesis of human leishmaniasis with regard to relapses and metastasis to mucosal tissues. Trauma-associated leishmanial lesions could be explored in depth in this animal model.

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***Plagiorhynchus (Prosthorhynchus) cylindraceus* (Goeze, 1782)  
Schmidt and Kuntz, 1966, from the Australian Bandicoots,  
*Perameles gunnii* Gray, 1838, and *Isodon obesulus* (Shaw, 1797)**

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**ABSTRACT:** *Plagiorhynchus (Prosthorhynchus) cylindraceus* (Goeze, 1782) Schmidt and Kuntz, 1966, usually parasitic in passerine birds, is here reported to be found encysted in the peritoneum of the bandicoot *Isodon obesulus* from Tasmania. Specimens recovered from the bandicoot *Perameles gunnii*, also collected from Tasmania, are the first reported from the intestine of a mammal. The origin of *P. cylindraceus* infection in Australian hosts is discussed.

The Peramelidae is an Australian and Malesian group of marsupials. Of the extant genera, 2 are exclusively Australian, *Isodon* and *Perameles*, with *Isodon obesulus* (Shaw, 1797) and *Perameles gunnii* Gray, 1838, occurring in Tasmania. Material from these 2 species was made available by the Mount Pleasant Laboratories of the Tasmanian Department of Agriculture for examination.

Acanthocephala from 2 *I. obesulus* individuals, from Glengarry (2 males, 4 females) and Hobart (1 juvenile), and 1 *P. gunnii* (34 males, 27 females) from Huonville, Tasmania, were found. The material from *I. obesulus* was encysted in the hosts' peritoneum. Similar findings reported from European insectivores have been assumed to be the result of accidental infection (Golvan, 1956).

The specimens from *P. gunnii* found in the small intestine of the host had a proboscis armature of 16 longitudinal rows of 13–14 hooks. The hooks increased in stoutness from the first 2 in each row through to the last 2 rootless, more delicate spines. Average female hook measurements in  $\mu\text{m}$  from the tip of proboscis to the base

were 62, 65, 69, 77, 81, 80, 83, 83, 84, 82, 77, 71, 69, 62. This arrangement and sizes of hooks fall within the description of *P. cylindraceus* (Golvan, 1956) and the measurements by Edmonds (1982) of specimens from Australian birds. The males were comparable in development to males described by Edmonds (1982) (see Table I), but none of the females had eggs.

*Plagiorhynchus cylindraceus* (Goeze, 1782) Schmidt and Kuntz, 1966, is an acanthocephalan usually parasitic in passerine birds. In reviews of the subfamily Plagiorhynchinae by Schmidt and Kuntz (1966) and subsequently of the genus *Plagiorhynchus* by Schmidt (1981), the separation of Old World from New World Acanthocephala, on biographical grounds, was challenged and the taxonomic status of *P. cylindraceus* as a cosmopolitan species established. Schmidt suggested that *P. cylindraceus* was originally a European species that became established in other regions as the range of suitable isopod intermediate hosts was extended.

The origin of Australian *P. cylindraceus* merits further examination. Material described by Edmonds (1982) is all from introduced birds but there is some evidence that native bird species have also become hosts for *P. cylindraceus*. Mawson et al. (1986) reported *P. cylindraceus* in a Tasmanian collection of *Gymnorhina tibicen*, the Australian magpie. Schmidt (1981) recognised juvenile specimens from "Australian pigeon" in material sent him from Victoria.

There is an earlier record of *P. cylindraceus*, from *Megalurus timorensis* and *Megalurus ga-*

TABLE I. Comparison of measurements of *Plagiorhynchus cylindraceus* from Australian birds and bandicoots.

Character	Hosts	
	<i>Turdus merula</i> Linn. and <i>Acridotheres tristis</i> Linn. (Edmonds, 1982)	<i>Perameles gunnii</i> Gray, 1938 (this paper)
Trunk	♂ 5.0–8.0 × 1.0–1.3 mm	5.5–7.5 (6.65) × 0.88–1.43 (1.25) mm
	♀ 6.0–13.0 × 1.4–1.9 mm	6.0–9.0 (7.4) × 0.98–1.33 (1.25) mm
Proboscis	♂ 0.93–1.0 × 0.22–0.27 mm	950–1,120 (1,019) × 210–270 (241)
	♀ 0.98–1.1 × 0.25–0.30 mm	960–1,150 (1,056) × 200–300 (264)
Proboscis receptacle	1.7–2.1 × 0.3–0.45	♂ 1,600–1,770 (1,724) × 230–340 (299)
		♀ 1,550–2,250 (1,859) × 300–390 (333.6)
Neck	0.14–0.20 mm long	♂ 180–350 (225) × 290–250 (303)
		♀ 150–250 (193) × 260–320 (295)
Lemnisci	Up to twice length of proboscis receptacle	♂ 1,225–2,900 (2,224) × 50–90 (70)
		♀ 1,675–2,730 (2,088.5) × 50–110 (71.1)
Testes	0.5–0.9 mm long	550–903 (771) × 350–510 (475.7)
		600–1,020 (699) × 280–530 (451)
Cement glands (length)		800–1,900 (1,355)
Female complex total length	1.2–1.9 mm	590–850 (722.75)

Measurements are in  $\mu\text{m}$  unless otherwise stated.

*lactotes* (Temm), the tawny grassbird (Marval, 1905), which has a northern distribution. Johnston and Deland (1929), however, cast doubt on the validity of the parasite identifications and suggested a re-examination of the parasite material. Although both Golvan (1956) and Yamaguti (1963) list *Megalurus* sp. as a host of *P. cylindraceus*, they do not indicate if the material was, in fact, re-examined. Therefore the only substantiated records of *P. cylindraceus* are from southeastern Australia.

It seems likely that *P. cylindraceus* reached Australia only after both the European hosts (isopod and bird) were introduced. *Porcellio scaber* and *Armadillidium vulgare*, potential isopod hosts, are found in Tasmania (Green, 1974). Establishment of infections in local host species would then follow. However, not only has *P. cylindraceus* established itself in Australian birds but also in Australian bandicoots. Bandicoots, predominantly insectivorous, use their forefeet to dig conical holes while searching for insects (adults and larvae) and earthworms. They also eat soft roots, berries, grass seeds, and rarely small rodents. Given the dietary preferences of *P. gunnii*, it is unlikely that infections with *P. cylindraceus* was the result of eating infected baby birds. It is more likely that the bandicoots were infected in the usual way by eating infected intermediate hosts.

Although the specimens were healthy in appearance, the lack of gravid females may be in-

dicative of either the period of time that the infection was established or that *P. gunnii* is an accidental host for *P. cylindraceus*.

This is the first record of *P. cylindraceus* in the intestine of a mammalian host. This record of *P. cylindraceus* in an Australian mammal is from southeastern Australia, the only region in which there is unequivocal evidence of *P. cylindraceus* in Australian birds.

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## Experimental Cryptosporidiosis in Fetal Lambs

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**ABSTRACT:** Fetal lambs were infected *in utero* with purified sporulated oocysts of *Cryptosporidium parvum* in order to study pathogenesis and host cellular response to the enteropathogen. Ileal loops (IL) of fetuses, 124-130 days of gestation, were inoculated with  $1-4 \times 10^6$  oocysts usually via cannulae in the abdominal wall of the ewe. Oocysts, both free and phagocytosed, were observed in the IL content as early as day 1 post-inoculation (PI). The percentage of oocysts phagocytosed by the host's polymorphonuclear neutrophils (PMN's) and mononuclear cells remained high up to day 13, the last day of examination. Numerous parasites were observed at days 6, 7, and 12 PI in the microvilli of the ileum with hypercellularity of the lamina propria, which consisted of a mixed infiltration of PMN's, mononuclear cells, including lymphoid cells, and a few eosinophils. Cytolysis and extrusion of epithelial cells, often heavily parasitized by various stages of the parasite, as well as inflammatory cells, were prominent in luminal contents. Germinal centers were prominent in mesenteric lymph nodes draining the infected loops by day 12 PI. Depletion of lymphoid cells was already present in Peyer's patches by day 4 PI.

Experimental cryptosporidiosis has been studied in several animals, including pathogen-free newborn lambs (Tzipori et al., 1980, 1981a, 1981b, 1981d, 1982; Angus et al., 1982). This report describes a fetal lamb model that allows observation of the cellular response to *Cryptosporidium parvum* in a fetal ileal loop *in utero* before the establishment of the normal intestinal flora of bacteria and yeast.

*Cryptosporidium parvum* oocysts, maintained by passage in newborn calves, were obtained from Dr. Harley Moon, NADC, Ames, Iowa. The procedure for preparation of oocysts has been described previously (Kim, 1987); only modifica-

tions will be cited. After oocysts were washed with 1% Clorox, they were placed over a discontinuous sucrose gradient of 3 densities, 1.18, 1.09, and 1.02 g/ml, and centrifuged at 2,300 rpm for 15 min at 4 C. Oocysts that were found primarily within the 1.09-g/ml density of sucrose were recovered and washed  $3 \times$  in cold phosphate-buffered saline (PBS). They were placed in a stirred cell and contaminants such as bacteria were filtered through a 3- $\mu$ m polycarbonate membrane (Nuclepore Corp., Pleasanton, California). The purified oocysts were recovered, washed in PBS, and centrifuged at 2,500 rpm for 10 min at 4 C. The pellet was resuspended in 5 ml of PBS, and an aliquot of 10  $\mu$ l was removed and counted in a hemocytometer. The desired number of oocysts for the inoculum was adjusted by volume. The inoculum was streaked onto MacConkey blood agar plates and into thioglycollate broth to test for the presence of aerobic and anaerobic bacteria. The cultures were free of any growth.

Ileal loops (IL) of 8 fetal lambs at 124-130 days of gestation were prepared. An incision was made along the linea alba of the pregnant ewe and a portion of the uterus containing the hind legs and posterior one-half of the fetus was exteriorized. An incision was then made in the uterine wall. After separating the placental layers, the amniotic sac was incised and the posterior one-half of the fetus exposed. An incision was made through the left abdominal wall of the fetus and the entire small intestine exteriorized. A small opening was made near the ileocecal junction and one end of a silastic cannula (inside diameter

0.2 cm, 100 cm long) inserted proximally, approximately 2 cm into the intestinal lumen, and secured tightly with silk sutures. A second cannula was placed anterior to the first (threaded distally into the lumen) creating an isolated loop of intestine approximately 50 cm in length. Cannulae were brought out through the abdominal wall of the fetus, the uterine and abdominal walls of the ewe, and the incision closed with sutures. Three-way stopcocks were placed on the ends of the exteriorized cannulae and secured high on the flank with silk sutures. The IL were inoculated with  $1-4 \times 10^6$  sporulated oocysts immediately following their surgical preparation either via the cannula or directly into the loops. A second ileal loop was created in the same fetus as a control and inoculated with PBS.

Samples of the intestinal content were obtained once on days 1, 2, 3, 5, 6, 7, 12, or 13 postinoculation (PI) by slowly infusing 55 ml of PBS containing penicillin G (to avoid external contamination) into the anterior cannula, and collecting 50 ml of perfusate from the posterior cannula if the volume of the inoculum were 5 ml. In 2 experiments, samples were collected on 3 separate days from the same IL; in all other experiments samples were collected only once on days cited above. The perfusate was centrifuged and the supernatant decanted down to 5 ml. The sediment was shaken and an aliquot of 5  $\mu$ l was removed for a simple monolayer smear that was stained by the modified acid-fast method and examined for oocysts under  $100\times$  oil immersion in at least 5 100-field samples. At necropsy on days 6, 7, and 12 PI, specimens of the IL were removed, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5  $\mu$ m, stained with hematoxylin and eosin, and 4-8 cross sections for each day examined for parasites under  $100\times$  oil immersion. Also, at necropsy, regional mesenteric lymph nodes from infected and uninfected areas of the ileum were removed and processed as for IL specimens.

On day 1 PI (24 hr after inoculation), oocysts were observed in the luminal content of the infected IL, along with intestinal villous epithelial cells and a few polymorphonuclear neutrophils (PMN's). In addition to free oocysts, those phagocytosed by PMN's were seen in the luminal content. At day 2, there were more free oocysts and those phagocytosed by mononuclear cells in addition to PMN's. Fewer oocysts were observed on day 3 than on previous days, although the percentage of oocysts phagocytosed by PMN's

and mononuclear cells was the same as at day 2. Fewer oocysts, epithelial cells, and phagocytes were seen in the lumen on day 12; however, almost half of the oocysts were phagocytosed. At day 13, there were still fewer oocysts but the percentage of phagocytosed oocysts was still relatively high. In experiments in which samples were obtained on 3 separate days from the same IL, the number of oocysts decreased with time, although the percentages of phagocytosed oocysts remained relatively constant. The contents from uninfected IL did not contain any oocysts and the numbers of epithelial and phagocytic cells were negligible.

A prominent finding at necropsy was distention of infected IL as compared to that of uninfected animals. For example, in 1 experiment, the inner diameter of the infected IL measured 7.17 mm as compared to 1.39 mm of the uninfected control 7 days PI. Also, mesenteric lymph nodes from infected fetuses as compared to those from uninfected controls were enlarged, particularly at 12 and 13 days PI.

When ileal sections were examined on days 6, 7, and 12, many parasites of different developmental stages were seen in the microvilli of both the villous tips and intestinal crypts. Microvilli were denuded and displaced by the invading parasites. Villous atrophy with fusion and epithelial cross-bridging between adjacent villi were observed. In addition to the presence of various stages of the parasite in the microvilli, there was hypercellularity of the lamina propria (Fig. 1), which consisted of a mixed infiltration of PMN's, macrophages, lymphoid cells, and a few eosinophils. Cytolysis and extrusion of epithelial cells into the lumen, many of them parasitized by various stages of the parasite, were prominent. In many instances, clumps of senescent epithelial cells appeared to be invaded by large numbers of the parasite. In some, sloughing of parasitized epithelial cells was accompanied by outpouring of inflammatory cells into the lumen. As expected, the control loops had no parasites in the microvilli and there was no cellular infiltration in the lamina propria.

Germinal centers were prominent in histological sections of regional mesenteric lymph nodes from infected fetuses on day 13 PI (Fig. 2); they were not observed in lymph nodes from uninfected fetuses. In Peyer's patches, depletion of lymphocytes was already evident by day 4 PI (Fig. 3 at day 6 PI), and by day 12 relatively few lymphoid cells remained.



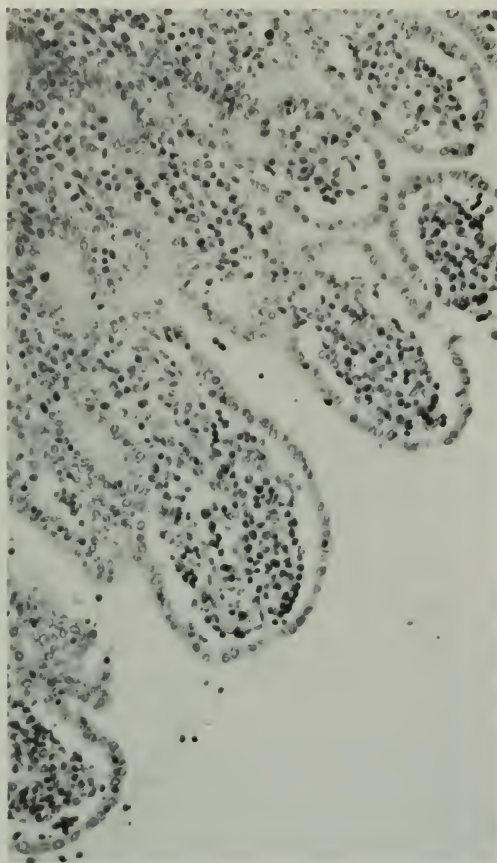


FIGURE 1. Villi of ileum of a fetus inoculated 6 days previously with  $1.7 \times 10^6$  sporulated oocysts. Note hypercellularity of the lamina propria.  $\times 150$ .

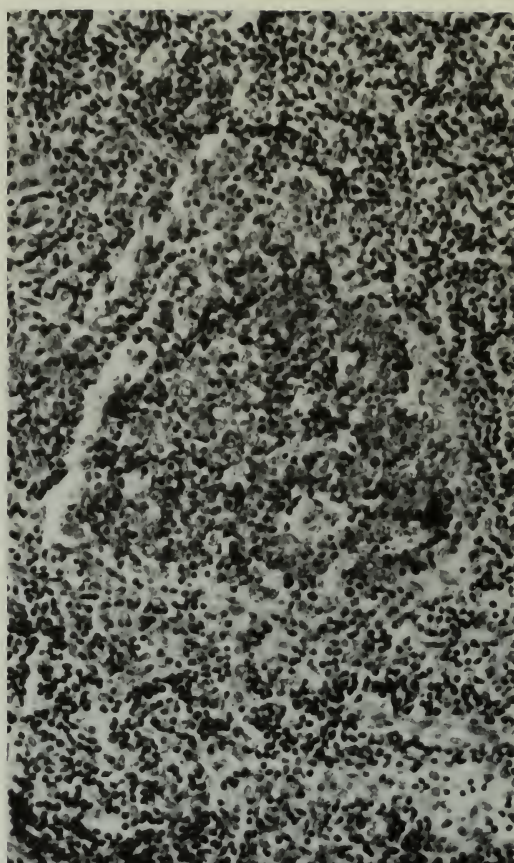


FIGURE 2. Regional mesenteric lymph node of a fetus inoculated 13 days previously with  $3.5 \times 10^6$  sporulated oocysts. Note the prominent germinal center.  $\times 150$ .

The present study demonstrates some of the pathological changes that occur in IL of fetuses infected *in utero* with *C. parvum*. Although oocyst counts were not quantitative, fewer oocysts were observed in the ileal content with time. The reduction of oocyst numbers with time in loops that were repeatedly sampled was most likely due to dilution. More importantly, we observed cellular activity early in the infection, as evidenced by the phagocytosis of the oocyst first by PMN's and later by mononuclear cells, that remained intense through 13 days PI. In fact, there appeared to be an increase in phagocytic activity with time.

The denudation of microvilli and their displacement by the parasite was easily seen under the light microscope, as reported earlier in hamsters (Kim, 1987). The acceleration of sloughing of epithelial cells, many of them heavily para-

sitized by the parasite, suggested that these senescent and presenescent cells were being destroyed by the parasite, as reported by others in calves (Pohlenz et al., 1978; Heine et al., 1984) and goat kids (Matovelo et al., 1984). The extrusion of parasitized epithelial cells, massive at times, was accompanied by outpouring of inflammatory cells, similar to what has been reported in piglets (Tzipori et al., 1981c). The accelerated destruction and loss of epithelial cells appear to be responsible for villous atrophy. More interesting and significant than the extrusion of parasitized epithelial cells and the villous atrophy was the intense hypercellularity of the lamina propria, which was infiltrated with PMN's and mononuclear cells, both macrophages and lymphoid cells, as well as eosinophils. Also, germinal centers of regional mesenteric lymph nodes with lymphopoiesis were observed in infected

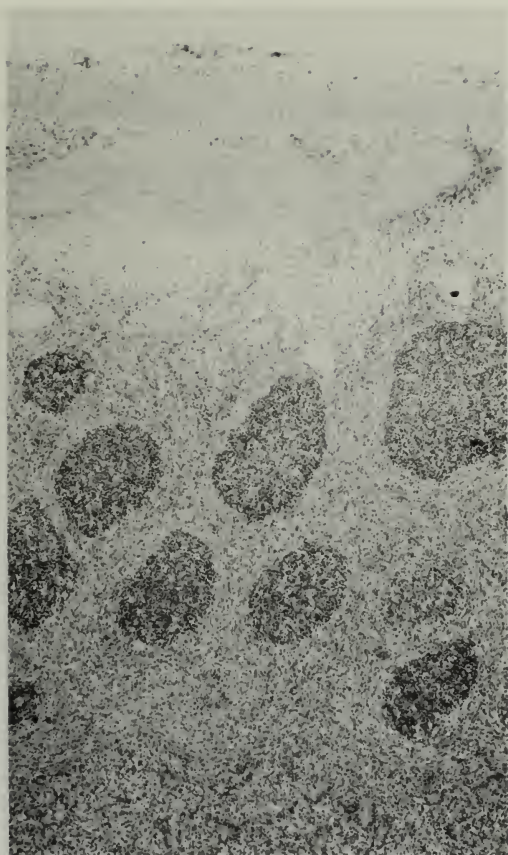


FIGURE 3. Peyer's patch from the ileum of a fetus inoculated 6 days previously with  $1.7 \times 10^6$  sporulated oocysts. Note sparsity of lymphoid tissue and lack of normal follicular structures.  $\times 40$ .

fetuses. These observations suggest that the parasite or perhaps some metabolic product of the parasite yet to be identified is a potent antigen. The intense cellular response that was observed, especially the presence of plasma cells and the stimulated germinal centers, shows that the immunologic state of the fetus at this stage of development is sufficiently competent to be capable of responding to the parasite. This may, in part, be responsible for the decrease in the number of oocysts in the luminal content with time.

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## *Ixodes dammini*: Evidence for Salivary Prostacyclin Secretion

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**ABSTRACT:** Pilocarpine-induced saliva of adult *Ixodes dammini* ticks contained abundant amounts ( $523 \pm 140$  ng/ml, mean  $\pm$  SE,  $n = 14$ ) of 6-keto-PGF<sub>1 $\alpha$</sub> , the stable degradation product of prostacyclin. This prostaglandin was identified by radioimmunoassay and reversed-phase chromatography. This activity may help tick feeding by preventing host hemostatic reactions, by increasing host blood flow at the tick feeding site, and by preventing leukocyte degranulation.

Ixodid ticks depend solely on their host's blood for growth and reproduction. Despite continuous exposure, some natural hosts fail to mount significant rejection reactions (Trager, 1939; Wikell and Allen, 1982). Evasion of a host's immune reactions may be accomplished by the ectoparasite's salivary pharmacological properties. Indeed, tick saliva contains antihemostatic, antiinflammatory, and immunosuppressive activities that may selectively prevent their natural host's tick rejection reactions (Ribeiro, 1987a).

Among the pharmacologically active components of tick saliva, prostaglandin E<sub>2</sub> was described both in the cattle tick, *Boophilus microplus* (Higgs et al., 1976), and in *Ixodes dammini* (Ribeiro et al., 1985). PGE<sub>2</sub> may increase tick feeding success by promoting hyperemia and consequently increasing the amount of blood to the tick's feeding site.

Other prostaglandins are also vasoactive and antihemostatic (Bach, 1982), but their presence in tick saliva is unknown. Because prostacyclin (PGI<sub>2</sub>) is a potent antiplatelet-aggregating and anti-mast cell-degranulating substance and could be potentially produced by tick salivary glands, we investigated whether the tick *I. dammini* was able to excrete this substance in saliva, as measured by its stable degradation product 6-keto-PGF<sub>1 $\alpha$</sub> .

Source of ticks and methods for obtaining pilocarpine-induced saliva were as in Ribeiro (1987b). Radioimmunoassays (RIA's) for PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub>  were performed as described previously. Specific antisera to PGE<sub>2</sub> and 6-keto-PGF<sub>1 $\alpha$</sub>  were provided by Dr. L. Levine, and have

been previously described (Axelrod and Levine, 1982; Ribeiro et al., 1985). Reversed-phase high performance liquid chromatography (HPLC) was performed with a Waters system (Waters Associates, Milford, Massachusetts) as described in detail elsewhere (Robinson et al., 1986).

To determine whether saliva of *Ixodes dammini* could contain prostacyclin, we radioimmunoassayed individual salivary samples for the presence of the stable derivative of prostacyclin, 6-keto-PGF<sub>1 $\alpha$</sub> . For comparison, we assayed the same samples for PGE<sub>2</sub>. In all 14 samples tested, both 6-keto-PGF<sub>1 $\alpha$</sub>  and PGE<sub>2</sub> were detected at concentrations that are pharmacologically significant, despite the large variation in results. Concentration of 6-keto-PGF<sub>1 $\alpha$</sub>  was  $593 \pm 140$  ng/ml, near 7 times the concentration of PGE<sub>2</sub> ( $80 \pm 38$  ng/ml) (Table I). The 6-keto-PGF<sub>1 $\alpha$</sub>  was further characterized by HPLC. Pooled tick saliva (200  $\mu$ l) was extracted with a reversed-phase column (Powell, 1982), and the eicosanoid fraction was subjected to HPLC. Fractions eluted from HPLC were reconstituted in isogel-tris buffer, pH 7.4, and radioimmunoassayed for 6-keto-PGF<sub>1 $\alpha$</sub> . Over 90% of the recovered activity by RIA was found in a symmetrical peak that cochromatographed with authentic 6-keto-PGF<sub>1 $\alpha$</sub>  standard. PGE<sub>2</sub>, detected by RIA, also cochromatographed with the PGE<sub>2</sub> standard. Therefore, tick saliva contains 6-keto-PGF<sub>1 $\alpha$</sub>  as defined by both RIA and chromatographic properties.

Despite the short half-life of prostacyclin at neutral pH (Salmon and Flower, 1982), it may be important during tick feeding. Ixodid ticks salivate at approximately 10-min intervals (Kemp et al., 1982), and thus, newly synthesized prostacyclin could be injected before all the previously injected substance had decayed to 6-keto-PGF<sub>1 $\alpha$</sub> . Prostacyclin may help tick feeding in several ways: (1) by preventing platelet aggregation (Salmon and Flower, 1982) it could prevent host hemostasis from occurring and thus keep blood from flowing from injured vessels to the tick's mouthparts; (2) by preventing mast cell

TABLE I.  $PGE_2$  and 6-keto-PGF $_{1\alpha}$  concentrations in individual samples of *Ixodes dammini* saliva.

Saliva sample	$PGE_2$ (ng/ml)	6-keto-PGF $_{1\alpha}$ (ng/ml)
1	104	902
2	113	1,265
3	51	1,565
4	540	1,349
5	193	414
6	2	173
7	7	134
8	1	35
9	17	156
10	17	405
11	6	73
12	12	265
13	50	512
14	9	86
$\bar{x} \pm SE$	$80 \pm 38$	$523 \pm 140$

degranulation (Bach, 1982) it could minimize both the reactions that may lead the host to groom or the edema that accompanies tick rejection reactions (Wikel and Allen, 1982); and (3) by inducing hyperemia (Bach, 1982) it could increase the amount of blood available for tick feeding. The multiple pharmacological actions of PGI $_2$  may all be of value for tick feeding success on its natural hosts.

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## BOOK REVIEW . . .

**Diagnostic Medical Parasitology**, by Lynne S. Garcia and David A. Bruckner. Elsevier, New York, Amsterdam, London. 1988. xii + 500 p.

Frequently parasitologists are asked "Which book should I take with me to the tropics as a practical laboratory and diagnostic guide?" Here is a new and exceptional candidate. It covers much the same ground as do other medical parasitology student-oriented texts—but with an emphasis on hands-on information regarding procedural techniques, culture, fixation, and preparatory methods, in keeping with the authors' extensive experience in this aspect of diagnostic parasitology.

Somewhat over half the book consists of brief treatments of disease-related information, including morphology, epidemiology, clinical disease, and therapy. Each chapter begins with a list of parasites to be reviewed. Key points in laboratory diagnosis are listed as a summary near the end of each chapter. References are well selected and some are current into 1987, though the general cutoff date appears to be 1986. The life cycle charts are reduced to the bare elements—a series of arrows connecting key stages, briefly stated—enough for a quick reminder. Relevant new chapters include one on parasitic infections and the compromised host; one on nosocomial and laboratory-acquired parasitic infections; another on serodiagnosis of parasitic diseases; and one on histological identification of parasites. Each of these chapters includes a lengthy tabular assemblage of information. In the histology chapter the table runs to over seven pages. Treatment is covered under the specific parasites, then collectively in a single chapter, both in tabular format and as a summary of each drug selected, including preparation, administration, toxicity, and contraindications. Some differences in the text from current recommendations should be noted: amebiasis drugs should be taken sequentially, not simultaneously; topical *Acanthamoeba* infection is not mentioned; ivermectin for onchocerciasis is alluded to in the text but not in the treatment chapter; repeat treatment for pinworm is not mentioned; praziquantel is not currently recommended for fascioliasis, whereas it should be included for *Hymenolepis nana* infection. For chloroquine-resistant malaria, Fansidar is not mentioned as a single-dose emergency therapy. The special danger of diethylcarbamazine in loiasis deserves emphasis.

The presentation is compact, consistently organized, and most of the material is current to the date of publication. A number of original tables condense and compare information on related groups of parasites, from morphology to epidemiology. Simplified algorithms combine presenting symptoms with successive steps leading to identification and treatment. Line

drawings of parasites by N. Kitamura and of arthropods and some parasites by Sharon Belkin are clear and helpful. A new single-page color plate shows the key morphological stages of *Plasmodium*. This is most welcome after the frequent use in other texts of the four Inez Demonet malaria color plates, which, though excellent, may give students the view that they are our sole standard.

Photographs range from fair to excellent, capped by a fine two-page series on *Babesia microti*. The now widely circulated kodachromes forming the "Pictorial Presentation of Parasites," collected and edited by Herman Zaiman, are much in evidence, and with good effect, as are a number of striking Armed Forces Institute of Pathology photos. Some unusual SEM photographs are included, such as one of Marietta Voge's prize *Cryptosporidium* shots.

A matter of some concern is the failure to acknowledge primary sources for some illustrative and tabular material. Such acknowledgments are often difficult in a basic text that must use a variety of sources. We will necessarily continue to have new texts with illustrations that span a number of earlier texts. Yet, when signed by the current artist, they are assumed by the student to be entirely original material. Not only is the historical record denied, but possible past inaccuracies are promulgated as current knowledge. To be able to discern the evolution of our knowledge and to identify some of the individuals responsible are among the joys of science and learning.

Part II of the book is an unusually detailed coverage of parasitological procedures, divided into 10 chapters. Among these are two unusual and useful chapters—one on artifacts confused with parasitic organisms; the second on quality control procedures, a valuable compendium for the parasitological laboratory in developing areas.

Additional tabular material is placed in an appendix: classification of parasites; geographic distribution of parasitic infections (arranged by continents); parasites listed by the organ infected; and various specialized lists, such as commercial reagents, and firms or individuals that can provide specimens and kodachromes. This is followed by a useful glossary and a detailed index.

As a compilation of current diagnostically based parasitological information of special value to the peripatetic parasitologist or the lab-based worker, this book is strongly recommended.

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## BOOK REVIEW . . .

**Handbook of Drugs for Tropical Parasitic Infections**, by Lars L. Gustafsson, Bjorn Beerman, and Yakoub Aden Abdi. Taylor & Francis Inc., London, New York, and Philadelphia. 1987. viii + 151 p. Softcover \$25.00; hardcover \$53.00.

The stated aim of this book is to supply information to clinicians on the pharmacology of drugs used in the treatment of tropical parasitic infections. This includes mechanism of action, pharmacokinetics, clinical trials, adverse effects, and dose schedule of each drug. The sources of information are handbooks of pharmacology/tropical medicine and scientific journals up to 1986.

The 151-page book is organized in sections of Preface, Abbreviations, Introduction, Drug Recommendations, Drugs (41 by name), and Index. The Preface includes the aim of the book, sources of information, acknowledgments, and references. The Introduction describes the global magnitude and historically important treatments of tropical diseases. In addition, there are general comments on clinical trials, bioavailability, and the response to those drugs covered in the text. Each disease (or parasite) is listed in tabular form as to whether it is protozoal or helminthic, along with the generic drug names of the various recommended chemotherapeutic agents, and pertinent comments. This is followed by a section of from 2 to 8 pages on each of 41 drugs, each listed alphabetically by name, manufacturer, chemical structure, physical properties, pharmacology, mechanism of action, pharmacokinetics, clinical trials, indications, (effects on) pregnancy and lactation, side effects, contraindications and precautions, interactions, dosage, preparations, and references, including proprietary names and manufacturers of several otherwise esoteric drugs used for treatment of some diseases. One advantage of such a listing is that it provides easy access to information about drugs available for use in the United States by physicians who may be completely unfamiliar with therapies for exotic parasitoses.

This concise book is useful especially to clinicians involved occasionally in the treatment of tropical diseases. Its small size and well-organized format make it a handy reference source. It contains many helpful facts such as: cure rates and percent side effects, the plasma half-life of specific drugs in pediatric and adult patients, whether the drug is "excreted" in the milk of lactating mothers, whether there is evidence of interaction with other agents, idiosyncratic reactions, ethnic differences in disposition of drug, and known antidotes. Drugs were chosen for the book with thought given to local availability, cost, efficacy, and safety; e.g., qinchaosu and its derivatives are included.

The book would be more useful (in the opinion of this reviewer) if certain statements were more carefully documented with literature references, but this may be debated as appropriate in a book of this length. Many drugs are simply passed off as "mechanism of action is not clear," and when documented, the reference is frequently out of date. For example, the discussion of the mechanism of action of drugs such as metronidazole, niclosamide, and antimony compounds could be explained, because considerable work has been done in these areas over the past 25 years. The authors correctly point out that hycanthone is hepatotoxic, teratogenic in mice, and mutagenic, but reports of the mutagenicity of metronidazole and niridazole are not addressed. The authors do state that, "Unlike other schistosomicides, praziquantel has not shown any mutagenic or carcinogenic effects." There is no mention of either schistosome resistance to oxamniquine or to the utility of employing praziquantel for treating *Metagonimus* infections. These omissions do not detract significantly, however, from the value of this text.

Recommended dosages of some of the drugs listed sometimes contrast with those given in other sources; e.g., adult doses of quinine combined with Fansidar are: quinine sulfate, 600 mg orally TID on days 1-3, and 500 mg sulfadoxine combined with 25 mg pyrimethamine (Fansidar®) TID for 1 day, on day 4 or 5. The Medical Letter (1988, 30: 15-24) recommends quinine sulfate, 650 mg TID  $\times$  3 days, plus sulfadiazine 500 mg QID  $\times$  5 days, plus pyrimethamine 25 mg BID  $\times$  3 days, for treatment of chloroquine-resistant *P. falciparum*. Therefore, it appears that one should consult more than this one source to determine which of several therapeutic regimens might be superior.

Therapy should always be appropriate to the clinical condition of the patient. There is no mention of the use of quinidine gluconate in situations where parenteral quinine is not available (Medical Letter, 1982, 24: 5-12). Also, the authors did not caution that i.v. quinine dihydrochloride is hazardous and must be given slowly with constant monitoring of pulse and blood pressure to avoid arrhythmia or hypotension.

In spite of these kinds of minor deficiencies, this text is potentially helpful both to the clinician who occasionally may be called on to treat tropical parasitic infections and the academician who is teaching students or planning health care programs. It is well worth the modest cost.

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## BOOKS RECEIVED (1988) . . .

- BERECZKY, M. C. (ed.). 1986. Advances in protozoological research. Proceedings of the 1st International Conference of Hungary on Protozoology and Memorial Session for Jozsef Gelei. Budapest, Hungary, 3-6 September 1985. Akademiai Kiado, Budapest, 550 p. (Symposia Biologica Hungarica Vol. 33)
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## CORRIGENDUM . . .

In the paper "The taxonomic status of *Actinocleidus* Mueller, 1937; *Anchoradiscus* Mizelle, 1941; *Clavunculus* Mizelle et al., 1956; *Anchoradiscoides* Rogers, 1967; *Synclithrium* Price, 1967 and *Crinicleidus* n. gen.: North American Ancyrocephalids (Monogenea) with articulating haptor bars," by Mary Beverley-Burton (72[1]: 22-44), please note the following corrections:

A. Page 22: In the Abstract, lines 11 and 12, "*Anchoradiscus* Mizelle, 1941, with *A. anchoradiscus* Mizelle, 1941, as type species, is redefined. *Actinocleidus triangularis* Summers, 1937, is, by the rule of tautonymy, regarded as a synonym of *A.*

*anchoradiscus*." should read "*Anchoradiscus* Mizelle, 1941, with *A. triangularis* (Summers, 1937) Mizelle, 1941, as type species, is redefined. *Anchoradiscus anchoradiscus* Mizelle, 1941, is regarded as a synonym of *A. triangularis*."

B. Page 29: In the Figure 2 caption, "*Anchoradiscus anchoradiscus* . . ." should read "*Anchoradiscus triangularis* . . ."

C. Page 30: In column 2, line 28 (last 3 lines of Remarks), "It appears that *A. anchoradiscus* occurs . . ." should read "It appears that *A. triangularis* occurs. . ."

## MAILING DATES FOR VOLUME 74, 1988

No. 1, February .....	mailed 12 April 1988
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## EDITORIAL ACKNOWLEDGMENTS (VOLUME 74, 1988) . . .

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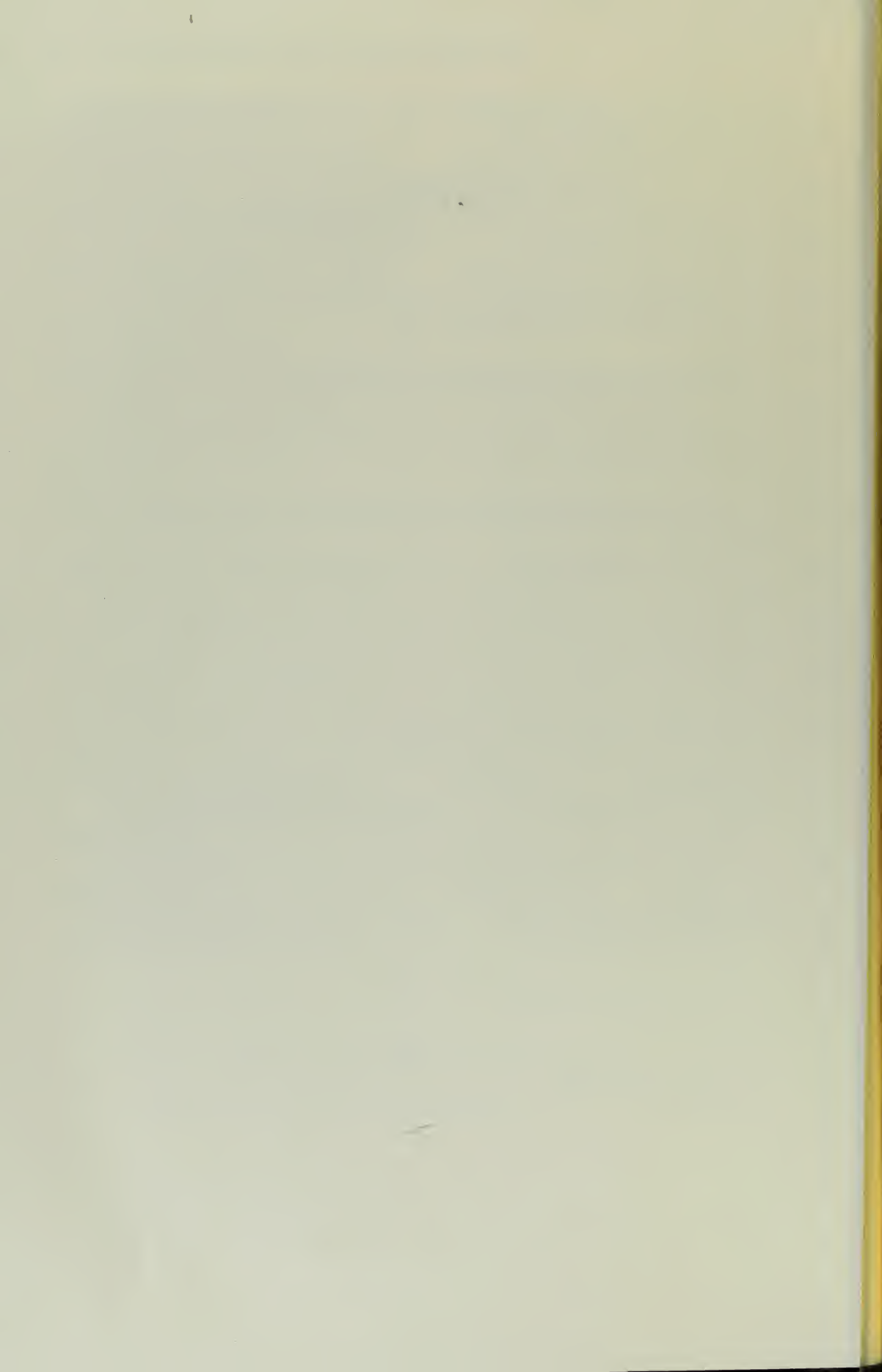
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